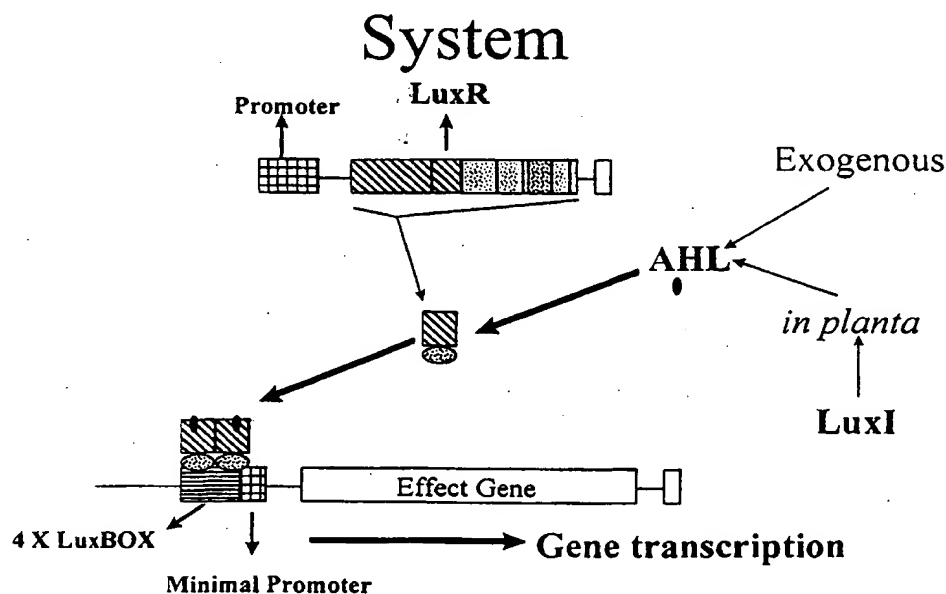




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(54) Title: GENE SWITCH



(57) Abstract

The present invention relates *inter alia*, to a method of initiating transcription of a target gene in a eukaryotic cell comprising: (a) providing a eukaryotic cell which is capable of producing a response protein; and (b) inserting into the genome of said cell a polynucleotide defining an inducible promoter sequence operably linked to and capable when induced of initiating transcription of said target gene; and (c) applying to said cell a chemical inducer capable of binding to said response protein whereby said chemical inducer binds to said response protein to form an inducing complex which binds to and induces said inducible promoter thereby initiating transcription of said target gene.

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GENE SWITCH

The present invention relates *inter alia*, to the induction of gene expression in a eukaryote by the application of a chemical inducer to the eukaryote and to materials
5 and methods for achieving induction. Such systems are referred to as "gene switches".

In particular, the present invention relates to a method of controlling expression of a target gene in a plant, animal or yeast.

Bacterial cells have the ability to respond to the surrounding environment.
10 The response to different environmental cues is essential for survival of bacteria. It is apparent that individual bacteria in a population are also able to sense the density and state of the local bacterial population (the "quorum") of which they are members. That is, an individual bacterium can detect the presence of like bacteria in the surrounding environment. Quorum sensing allows bacteria to synchronise growth,
15 development which when a minimal population level is reached initiates a concerted response from the population.

In the case of *Photobacterium fischeri*, *N*-(3-oxohexanoyl)-L-homoserine lactone or autoinducer regulates bioluminescence in a cell density-dependent manner. There are two main genes in the *lux* operon of *P. fischeri* involved in the signal
20 production and signal detection. *LuxI* is the gene involved in the biosynthesis of the homoserine lactone but the mechanism by which this takes place is unclear. It has been proposed that *S*-adenosylmethionine and coenzymeA or the acyl carrier protein adduct of 3-oxohexanoic acid are substrates for the *luxI* gene in *P. fischeri*.

In bacteria, the autoinducer regulates expression of the *luxI* gene and thus
25 creates a positive autoregulation of autoinducer synthesis. *LuxR*, the response regulator or autoinducer receptor, is a protein involved in responding to the presence of *N*-(3-oxo)hexanoyl-L-homoserine lactone (OHHL) in *P. fischeri*. At the C-terminal end *LuxR* contains a DNA binding domain and a transcriptional activator. *LuxR* C-terminal end shows amino acid homology to transcriptional activators known
30 as the two component environmental-sensing systems such as UhpA, FixJ and NarL. *LuxR* is thought to interact with DNA as a homodimer to a palindrome within the *luxI* operator sequence termed *lux* box. The N-terminal end of the protein is called the

receptor module as it has no similarity to the two-component environmental sensing systems.

Quorum sensing systems are found in other bacteria and may be activated by different homoserine lactones. Such is the case in *P. aeruginosa* PAO1, *LasI* directs the synthesis of the autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), which activates the positive transcriptional activator, LasR (Winson et al., 1995). Moreover, in the same *P. aeruginosa*, PAO1, a second signalling pathway termed *vsm* containing *vsmR* and *vsmI* genes was isolated. The *vsmI* gene product is involved in production of *N*-butanoyl-L-homoserine lactone (BHL) and *N*-hexanoyl-L-homoserine lactone (HHL). These compounds are present in the spent culture supernatants of *P. aeruginosa* and when either BHL or HHL to PAN067, a pleiotropic *P. aeruginosa* mutant unable to synthesize either of these autoinducers, restored elastase, chitinase, and cyanide production (Winson et al., 1995). Other evidence suggesting the presence of different homoserine inducers in one species has recently been observed in *Vibrio anguillarum* (Milton et al., 1997) and *P. aeruginosa* (Pesci et al., 1997). Furthermore, Table 1, shows examples of characterised systems where different compounds are known to be inducers of different receptor molecules in different bacteria.

Table 1.

Organism	Signal generator	Response Regulator	Signal Molecule	GenBank Accession number	References
<i>Aeromonas hydrophila</i>	AhyL	AhyR	unknown	X89469	
<i>Agrobacterium tumefaciens</i>	TraI	TraR	N-(3-oxo)-octanoyl-L-homoserine Lactone (OOHL)	L17024, L22207	Fuqua and Winans, 1994; Hwang <i>et al.</i> , 1995
<i>Chromobacterium violaceum</i>	CviI	CviR	N-hexanoyl-L-homoserine lactone (OHL)		Winson, <i>et al.</i> , (1994)
<i>Enterobacter agglomerans</i>	EagI	unknown	N-(3-oxo)hexanoyl-L-homoserine lactone (OHHL)	x74300	Swift <i>et al.</i> , 1993
<i>Erwinia carotovora</i> <i>sudsp carotova</i>	Carl	CarR	OHHL	U17224, X72891, X74299, X80475	McGowan <i>et al.</i> ., 1995
<i>Erwinia stewartii</i>	Esal	EsaR	OHHL	L32183, L32184	Beck von Bodman and Farrand, 1995

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<i>Escherichia coli</i>	unknown	SdiA	unknown	Xo3691	Sitnikov <i>et al</i> 1995
<i>Photobacterium fischeri</i>	LuxI	LuxR	OOHL, OOHHL	M19039, M96844, M25752	Meighen, 1994; Devine <i>et al</i> , 1988
<i>Pseudomonas aeruginosa</i>	LasI	LasR	N-(3-oxo)-dodecanoyl-L-homoserine lactone (OdDHL)	M59425	Winson <i>et al</i> 1995; Jones <i>et al</i> , 1993; Pearson <i>et al.</i> , 1995.
	VsmI	vsmR	N-butanoyl-L-homoserine lactone (BHL), HHL	L08962, U11811, U15644	Winson <i>et al.</i> , 1995; Williams <i>et al</i> , 1996 Latifi <i>et al</i> 1995; Ochsner and Reiser, 1995.
<i>Pseudomonas aureofaciens</i>	PhzI	PhzR	unknown	L32729, L33724	Wood and Piersen, 1996
<i>Rhizobium leguminosarum</i>	unknown	RhiR	N-(3-hydroxy)-tetradecanoyl-L-homoserine lactone (HtDeHL)	M98835	Fuqua <i>et al.</i> , 1994; Gray <i>et al.</i> , 1996.
<i>Serratia liquefaciens</i>	SwrI	unknown	BHL	U2823	

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<i>Aeromonas hydrophila</i>	ahyI	ahyR	BHL		Swift et al., 1997
<i>Aeromonas salmonicida</i>	AsaI	unknown	BHL, N-hexanoyl-L-homoserine lactone		Swift et al., 1997
<i>Vibrio anguillarum</i>	vanI	vanR	N-(3-oxo-decanoyl)-L-homoserine lactone (ODHL)		Milton et al., 1997
<i>Vibrio harveyi</i>	LuxLM	LuxN	N-(3-hydroxy)-butanoyl-L-homoserine lactone (HBHL)	L13940	Meighen, 1994; Bassler et al., 1994.
<i>Yersinia enterocolitica</i>	YenI	YenR	OHHL, HHL	X76082	Throup et al., 1996.

The present invention therefore seeks to provide *inter alia*, methods and materials for the induction of gene expression in eukaryotes by the application of chemical inducers to the eukaryote.

- 5 According to the present invention there is provided a method of initiating transcription of a target gene in a eukaryotic cell comprising:
- (a) providing a eukaryotic cell which is capable of producing a response protein;
 - (b) inserting into the genome of said cell a polynucleotide defining an inducible promoter sequence operably linked to and capable when induced of initiating
 - 10 transcription of said target gene; and
 - (c) applying to said cell a chemical inducer capable of binding to said response protein whereby said chemical inducer binds to said response protein to form an inducing complex which binds to and induces said inducible promoter thereby
 - 15 initiating transcription of said target gene.

- 15 The eukaryotic cell may already contain the mechanisms to produce the said response protein or may alternatively be provided with them by inserting into the said cell, a polynucleotide which provides for the production of the response protein using techniques well known within the art. The inducible promoter for use in the above mentioned method may comprise the nucleotide sequence depicted as SEQ ID No. 2
- 20 and the response protein may comprise the amino acid sequence depicted in SEQ ID No. 16. Alternatively the inducible promoter may comprise a functional variant such as a polynucleotide which is the complement of one which binds to SEQ ID No. 2 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength
- 25 citrate buffered saline containing 0.1% SDS wherein the said polynucleotide is still capable of acting as an inducible promoter upon binding with the said inducing complex. In particular the response protein for use in the method of the present invention may be encoded by the polynucleotide comprises the sequence depicted in SEQ ID No. 5. Alternatively the polynucleotide encoding the said response protein
- 30 may comprise the complement of one which binds to SEQ ID No. 5 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline

containing 0.1% SDS wherein the said polynucleotide still encodes a protein which is capable of forming an inducing complex with the said chemical inducer. It is particularly preferred that the polynucleotide defining the inducible promoter according to the present invention contains the region depicted as SEQ ID No. 21.

- 5 The method of present invention is particularly applicable to initiating transcription in cells of plants, more particularly in the cells of: melons, mangoes, soybean, cotton, tobacco, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tomato, alfalfa, lettuce, maize, wheat, sorghum, rye, bananas, barley, oat, turf grass, forage grass, sugar cane, pea, field bean, rice, pine, poplar, apple, peaches, grape, strawberries,
- 10 carrot, lettuce, cabbage, onion, citrus or nut plants. In particular the method of the present invention may be used to initiate transcription in a variety of tissues, including roots, leaves, stems and reproductive tissues. The chemical inducer which may be used in the above mentioned method is N-(3-oxo)hexanoyl-L-homoserine lactone or a functional equivalent thereof and may be applied to the plant cell as part of an
- 15 agriculturally acceptable formulation. Alternatively the chemical inducer may be produced within the plant by inserting into the genome of the plant a polynucleotide encoding a protein which provides for the production of the chemical inducer within the plant. This polynucleotide may, for example, be under the transcriptional control of a constitutive promoter, a gene switch (such as the *alcA/R*, *Heliothis* ecdysone and
- 20 GST-27 gene switch), wound inducible, tissue or temporal promoters. The advantage of producing the chemical inducer *in planta* is that there is no need to spray or exogenously treat plants to induce gene expression.

- Further alternative inducible promoters, response proteins and chemical inducers which may be used in the method of the present invention are referenced in
- 25 Table 1. For example, the promoter sequence or a part thereof may obtainable from the *vanI* gene of *Vibrio anguillarum* and the response protein encoded by the *vanR* gene may be used with the chemical N-(3-oxo-decanoyl)-L-homoserine lactone (ODHL) or a functional variant thereof.

- The polynucleotide encoding the said response protein in the method referred
- 30 to above may be bounded by a promoter and a terminator sequence and in particular the promoter may be inducible such as the *Alc A/ R* switch system, the GST switch system and the ecdysone switch. Alternatively the promoter may be constitutive such

as cauliflower mosaic virus 35S/19S, maize Ubiquitin and *Arabidopsis* Ubiquitin 3 or may be developmentally regulated specific promoter such as those controlling expression of gene required during seed formation, germination such as cysteine proteinases (as specified in our International Publication No WO 97/35983) and
5 malate synthase.

The target gene in the method referred to above may be any gene of interest, for example, β -glucuronidase; *Bacillus thuringensis* toxin; barnase or barstar.

In a further aspect of the present invention there is provided a method of providing plants containing an inducible target gene comprising:

- 10 (a) inserting into a plant cell which cell provides for production of a response protein a polynucleotide defining an inducible promoter sequence operably linked to said target gene; and (b) regenerating morphologically normal fertile plants thereof; and (c) applying to the population of regenerants a chemical inducer or a functional variant thereof capable of binding to said response protein, whereby said chemical inducer
15 binds to said response protein to form an inducing complex which binds to and induces said inducible promoter thereby initiating transcription of said target gene; and (d) selecting those plants which are expressing the said target gene. The inducible promoter sequence referred to in the preceding paragraph may comprise the nucleotide sequence depicted as SEQ ID No. 2 or a functional variant thereof and the
20 response protein comprises the amino acid sequence depicted as SEQ ID No 16 or a functional variant thereof and the said chemical inducer is N-(3-oxo)hexanoyl-L-homoserine lactone or a functional equivalent thereof. In particular the inducible promoter sequence may contain the nucleotide sequence depicted as SEQ ID No. 10 or a functional variant thereof and the response protein may comprises the amino acid
25 sequence depicted as SEQ ID No 17 or a functional variant thereof and the said chemical inducer may be N-(3-oxo)dodecanoyl-L-homoserine lactone or a functional equivalent thereof. Alternatively, the said inducible promoter sequence may contain the nucleotide sequence depicted as SEQ ID No. 12 or a functional variant thereof and the response protein may comprise the amino acid sequence depicted as SEQ ID
30 No 18 or a functional variant thereof and the said chemical inducer may be N-(3-oxo)octanoyl-L-homoserine lactone or a functional equivalent thereof.

The present invention also provides plants produced according to the method of the preceding paragraph which plants may be selected from the group consisting of: melons, mangoes, soybean, cotton, tobacco, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tomato, alfalfa, lettuce, maize, wheat, sorghum, rye, bananas, barley, oat, turf grass, forage grass, sugar cane, pea, field bean, rice, pine, poplar, apple, peaches, grape, strawberries, carrot, lettuce, cabbage, onion, citrus or nut plants.

The method employed for transformation of plant cells is not especially germane to the present invention and any method suitable for the target plant may be employed. For example, transgenic plants are obtained by regeneration from the transformed cells. Numerous transformation procedures are known from the literature including agroinfection using *Agrobacterium tumefaciens* or its Ti plasmid, electroporation, microinjection or plants cells and protoplasts, microprojectile transformation. All of these methods are well known within the art. The present invention may also be applied to any plant for which transformation techniques are, or become, available.

In a further aspect of the present invention there is provided a DNA construct which comprises a first polynucleotide region which comprises an inducible promoter sequence operably linked to and capable when induced of initiating transcription of a target gene and a second polynucleotide region which provides for the production of a response protein wherein upon contact of the said response protein with a chemical inducer the said response protein binds the chemical inducer to produce an inducer complex which binds the said inducible promoter sequence thereby initiating transcription of the said target gene. The said first polynucleotide of the DNA construct may comprise the nucleotide sequence depicted as SEQ ID No. 2 or a functional variant thereof and the second polynucleotide region may comprise a polynucleotide which encodes the amino acid sequence depicted as SEQ ID No. 16 or a functional variant thereof. Alternatively the said first polynucleotide may comprise the nucleotide sequence depicted as SEQ ID No. 10 or a functional variant thereof and the second polynucleotide region may comprise a polynucleotide which encodes the amino acid sequence depicted as SEQ ID No. 17 or a functional variant thereof. Alternatively, the said first polynucleotide may comprise the nucleotide sequence

depicted as SEQ ID No. 12 or a functional variant thereof and the second polynucleotide region may comprise a polynucleotide which encodes the amino acid sequence depicted as SEQ ID No. 18 or a functional variant thereof. The said second polynucleotide region referred to above may comprise a promoter operably linked to the polynucleotide encoding the said response protein. The said promoter may be inducible and constitutive or developmentally controlled.

The present invention still further provides the use of a DNA construct referred to above in the production of a plant containing a target gene of which the expression may be controlled by the application of a chemical inducer to the said plant.

10 In a further aspect of the present invention there is provided a method of screening compounds in a bioassay comprising applying an amount of a chemical to a plant which plant contains a polynucleotide comprising an inducible promoter operably linked to a region encoding a reporter protein and which plant is also capable of producing a response protein and testing the said plant for the production of the said reporter protein. The chemical could be applied to the plant and the activity of the reporter gene monitored for e.g. improved activity, mobility or stability or to assess if the chemical had an inhibitory effect on the response protein which resulted in a decrease in activity of the reporter gene.

20 In a further aspect of the present invention there is provided a method of selectively controlling pests in a field which field comprises crop plants and pests wherein the plants are those obtained according to the methods referred to above and the said target gene encodes a target protein which is capable of controlling the said pests said method comprising applying to the plants an amount of a chemical inducer which is sufficient to bind to the said response protein to produce the said inducing complex which is capable of initiating transcription of the target gene which provides for the production of the target protein in an amount which is sufficient to control the said pests.

25 In a further aspect of the present invention there is provided a method of providing a plant which contains a target gene which is inducibly controlled comprising:

30

- (a) inserting into a first plant cell a polynucleotide comprising a first inducible promoter operably linked to a target gene and regenerating a first morphologically normal fertile plant therefrom;
- (b) inserting into a second plant cell a polynucleotide comprising a promoter
5 operably linked to a region encoding a response protein which is capable of binding to a chemical inducer to produce an inducing complex which is then capable of binding the said inducible promoter to allow for the initiation of transcription of the said target gene and regenerating a second morphologically normal fertile plant therefrom;
- (c) cross pollinating said first plant with the said second plant or said second plant
10 with said first plant and harvesting the seed therefrom;
- (d) growing said seed and applying to the resultant plants an amount of said chemical inducer which provides an inducing complex capable of binding the said inducible promoter to allow for the initiation of transcription of the said target gene.

15 The term "functional variant" with respect to a polynucleotide encoding the inducible promoter of the present invention includes variant sequences which are the complement of a sequence which hybridises to the inducible promoter sequence at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength
20 citrate buffered saline containing 0.1% SDS and which are still capable of acting as an inducible promoter.

The term "functional variant" with respect to the response protein includes variant proteins obtained by conservative substitutions within the amino acid sequence which substitutions do not significantly adversely affect the ability of the
25 response protein to bind the chemical inducer. In particular substitutions may be made between the following amino acid groups viz.

- (a) Alanine, Serine, Glycine and Threonine
- (b) Glutamic acid and Aspartic acid
- (c) Arginine and Lysine
- 30 (d) Isoleucine, Leucine, Valine and Methionine
- (e) Phenylalanine, Tyrosine and Tryptophan

The term "transgenic" in relation to the present invention does not include a wild type regulator promoter in its natural environment in combination with its associated functional gene in its natural environment.

The term "target gene" with reference to the present invention means any gene
5 of interest. A target gene can be any gene that is either foreign or natural to the eukaryote in question.

The term "construct" - which is synonymous with terms such as "cassette", "hybrid" and "conjugate" - includes a target gene directly or indirectly attached to the regulator promoter, such as to form a cassette. An example of an indirect attachment
10 is the provision of a suitable spacer group such as an intron sequence intermediate the promoter and the target gene. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. Such constructs also include plasmids and phage which are suitable for transforming a cell of interest.

The term "expression system" means that the system defined above can be
15 expressed in an appropriate organism, tissue, cell or medium. The system may comprise one or more constructs and may also comprise additional components that ensure to increase expression of the target gene by use of the regulator promoter.

One possible use of the inducible promoters of the present invention is in the control of male sterility. The anther is the site of male reproductive processes in
20 flowering plants. It is composed of several tissues and cell types and is responsible for producing pollen grains that contain the sperm cells. The tapetum is a specialised tissue which plays a critical role in pollen formation. It surrounds the pollen sac early in pollen development, degenerates during the latter stages of development and is not present in an organised form in the mature anther. The tapetum produces a number of
25 compounds which aid pollen development or are incorporated into the pollen outer wall and it has been demonstrated that many of the natural male sterility mutations have impaired tapetum differentiation or function.

Tapetal tissue is therefore critical to the formation of functional pollen grains. A number of genes have been identified and cloned that are specifically expressed in
30 tapetal tissue. They include Osg6B, Osg4B (Tsuchiya *et al.* 1994, Yokoi, S *et al.* 1997), pE1, p T72 (WO9213957), p CA55 corn (WO92/13956), TA29, TA13, (Seurinck *et al.* 1990), RST2 corn (WO9713401), MS14,18,10 and A6, A9

from *Brassica napus* (Hird *et al.* 1993). Anther specific clones have been isolated from a number of species Bp4A and C (Albani *et al.* 1990), chs petunia (Koes *et al.* 1989), rice (Xu *et al.* 1993, Zou *et al.* 1994), amongst others. In higher plants the female reproductive organ is represented by the pistil, composed of the ovary, style and stigma. The gynoecium has been shown to contain up to 10,000 different mRNAs not present in other organs (Kamalay and Goldberg 1980). These include regulatory genes responsible for controlling pistil development as well as "downstream" ones encoding proteins associated with differentiated cell types in the pistil. Genes governing self-incompatibility and their homologues are one class of gene with pistil predominant expression patterns (Nasrallah *et al.* 1993). Other cloned genes which are applicable as target genes in the present invention include β glucanase (Ori *et al.* 1990), pectate lyase (Budelier *et al.* 1990) and chitinase (Lotan *et al.* 1989) which are expressed in the transmitting tissue and a proteinase inhibitor (Atkinson *et al.* 1993) which are expressed in the style. Others are pathogenesis related or are homologues of genes involved in the cleavage of glycosidic bonds. These enzymes may facilitate pollen tube growth by digesting proteins in the tissue through which the pollen tube grows. A number of female sterile mutants have been identified in *Arabidopsis*. For example, sin1 (short integument) (Robinson-Beers *et al.* 1992) and bell (bell) (Robinson-Beers *et al.* 1992) affect ovule development. In the short integument a mutation blocks megasporogenesis at the tetrad stage (Elliot, R.C., *et al.* 1996, Klucher, K.M., 1996). A lethal ovule 2 mutation has been observed but not cloned in maize (Nelson *et al.* 1952). Pistil specific basic endochitinases have been cloned from a number of species (Ficker *et al.* 1997, Dzelzkalns *et al.* 1993, Harikrishna *et al.* 1996, Wemmer *et al.* 1994) and extensin-like genes have been shown to be expressed in the styles of *Nicotiana glauca* (Chen C-G, *et al.* 1992). The following are ovule specific clones ZmOV23,13, (Greco R., *et al.* unpublished), OsOsMAB3A (Kang H.G., *et al.* 1995), ZmZmM2 (Theissen G., *et al.* 1995) and stigma specific stig1 (Goldman, M.H *et al.* 1994), STG08, STG4B12 (EP-412006-A). Mariani *et al.* used the promoter from the STIG1 gene to drive expression of barnase in the stigmatic secretory zone.

In summary then, the present invention therefore provides a gene switch which is operably linked to a foreign gene or a series of foreign genes whereby expression of

said foreign gene or said series of foreign genes may be controlled by application of an effective exogenous inducer. The gene switch of the present invention, therefore, when linked to an exogenous or foreign gene and introduced into a eukaryote by transformation, provides a means for the external regulation of expression of that foreign gene.

It is possible to use one, two or more of these inducible promoter regions according to the present invention to activate different processes in plant cells, thereby obtaining a plant which will have multiple inducible cassettes all controlled by for example, different homoserine lactones. Also, a plant may contain, for example, an inducible promoter according to the present invention in conjunction with other switch type mechanisms examples of which include inducible promoters include the Alc A/ R switch system described in International Publication No. WO. 93/21334, the GST switch system described in International Publication Nos WO 90/08826 and WO 93/031294 and the ecdysone switch described in our International Publication No. WO 96/37609.

The methods and products of the present invention may also be used to control expression of foreign proteins in eukaryotes such as yeast and mammalian cells. Many heterologous proteins for different applications may be produced by expression in such eukaryotic cells. The present invention is advantageous in that it provides control over the expression of foreign genes in such cells. It also provides a further advantage, particularly in yeast and mammalian cells, where accumulation of large quantities of a heterologous protein can damage the cells, or where the heterologous protein is damaging such that expression for short periods of time is required in order to maintain the viability of the cells. The inducible system of the present invention also has applicability in gene therapy as it allows the timing of the therapeutic gene to be controlled. The present invention is therefore not only advantageous in transformed mammalian cells but also to mammals *per se*. Furthermore, the present invention may be used to switch on genes which produce potentially damaging or lethal proteins. Such a system may be employed in the treatment of cancer in which cells are transformed with genes which express proteins which are lethal to the cancer. The timing of the action on such proteins on the cancer cells may be controlled using the switch of the present invention.

Various preferred features and embodiments of the present invention will now be described by way of non-limiting examples with reference to the accompanying Figures of which:-

5

Figure 1 shows a schematic representation of the general structure of the response regulator protein of the bacterial quorum sensing system.

Figure 2 shows a schematic representation of the homoserine lactone gene switch system.

10 Figure 3 is a plasmid map of the reporter gene construct, p221.9lux6.

Figure 4 is a plasmid map of p221.9lux2.

Figure 5 Plasmid map of p221.9lux3.

Figure 6 Plasmid map of p221.9luxC.

Figure 7 Plasmid map of p221.9Lpro.

15 Figure 8 Depicts the expression vector containing the LuxR gene in the pDH51LuxR plasmid.

Figure 9 Shows the expression cassette containing the enhanced N-terminal fusion protein of SV40-NLS-Gal4-LuxR in the plasmid pDH51SVLuxR.

Figure 10 Shows the expression cassette containing the enhanced C-terminal fusion of

20 LuxR-SV40-NLS-Gal4 in the plasmid DH51LuxRSV.

Figure 11 is the plasmid map of p221.9lasbox. Containing one copy of the LasR Box.

Figure 12 is a plasmid map of the transient expression construct containing LasR, pSinLASR.

Figure 13 expression plasmid pFunLuxR for expression in monocotyledon

25 protoplasts.

Figure 14 expression plasmid pFunLasR for expression in monocot protoplasts.

Figure 15 expression plasmid pFunTraR for expression in monocot protoplasts.

Figure 16 reporter plasmid p221.9trabox1 containing tra box sequence 1 described by Zhu and Winans, (1999)

30 Figure 17 reporter plasmid p221.9trabox2 containing tra box of sequence 2 described by Zhu and Winans, (1999).

Figure 18 is a plasmid map of binary vectors containing the effector and reporter cassettes, pAHL1.

Figure 19. is a plasmid map of binary vectors containing the effector and reporter cassettes, pAHL2.

5 Figure 20. is a plasmid map of binary vectors containing the effector and reporter cassettes, pAHL3.

Figure 21 is a plasmid map of binary vector containing *luxI* gene, pBDHELI.

Figure 22 is a plasmid map of pSB401 containing the Lux operon.

Figure 23 shows the root of a transgenic tobacco plant that was grown in tissue culture
10 under aseptic conditions was placed on an LB agar plate and overlain with top agar containing *E. coli* with the plasmid pSB401 (Figure 22). This strain expresses the lux operon and will bioluminesce in response to OHHL and HHL. Bioluminescence in the region around the root was clearly seen with the naked eye.

Figure 24. Shows a leaf of a transgenic tobacco plant that was grown in tissue culture
15 under aseptic conditions. The leaf was excised from the plant and placed on an LB agar plate and overlaid with top agar containing *Chromobacterium violaceum* indicator strain. The violet colour detected represent the induction of the indicator stain gene showing the extrusion of OHHL from the plan tissue into the bacterial medium.

20 Sequences

SEQ ID No. 1 is the *LuxI* box promoter region.

SEQ ID No. 2 is the *LuxI* promoter region.

SEQ ID No. 3 & 4 are the *LuxRBam*h1 fragment.

SEQ ID No. 5 & 6 are the *LuxR* sequence/protein sequence.

25 SEQ ID No. 7 & 8 are the NVLuxR fusion flanked by BamHI and PstI sites/protein sequence.

SEQ ID No. 9 & 10 are the LuxRNV sequence/protein sequence.

SEQ ID No. 11 is a TraR1 fragment.

SEQ ID No. 12 is the TraR2 fragment.

30 SEQ ID No. 13 is the LasBox1 region.

SEQ ID No. 14 is the LasBox2 region.

SEQ ID No. 15, 16, 17 and 18 are the TraBox1,2,3,4 regions respectively.

SEQ ID No. 19 is the *LuxR* response protein sequence.

SEQ ID No. 20 is the *LasR* response protein sequence.

SEQ ID No. 21 is the *TrasR* response protein sequence.

SEQ ID No. 22 & 23 are the open reading frames of *LasR* and *TraR* respectively.

5 SEQ ID No.24 is the *Lux* Box promoter region.

Example 1. Transient expression of *LuxR* and reporter plasmid in Tobacco mesophyll protoplasts.

Preparation of Reporter gene expression cassette.

10 Six copies of the 20bp palindromic *lux* box sequence will be fused upstream of the -60CaMV minimal promoter which in turn is fused to the reporter gene GUS. The palindrome of sequence 5' GATCACCTGTACGATCGTACAGGT 3' (Sequence ID. 1) was self annealed and introduced into a *Bam*HI pBluescript vector. Sequence determination of a number of recombinants lead to the identification of a plasmid with
15 6 copies of the palindrome. The identified plasmid was digested with *Hind*III and *Sa*I to release the in 6 copies of the palindrome and introduced into a *Hind*III/*Sa*I p221.9 vector. p221.9 plasmid contains a -60CaMV minimal promoter fused to GUS downstream of the *Hind*III and *Sa*I unique cloning sites. A recombinant plasmid was identified and named p221.9lux6 (Figure 3). The same oligonucleotide was used to
20 generate p221.9LuxR2 (Figure 4), p221.9LuxR3 (Figure 5) and p221.9LuxC (Figure 6). p221.9lpro was generated using the bacterial promoter containing the *Lux* box sequences (Figure 7 Sequence ID 2).

Preparation of *LuxR* expression vector.

25 The *lux* receptor was altered at both ends of the coding sequence. At the 5' end a plant Kozac consensus sequence was placed with an *Nco*I site at the ATG start of the coding sequence. Upstream from the Kozac consensus sequence a *Bam*HI unique site was introduced using PCR. The sense oligonucleotide was luxrbamh1 5' CCCGGATCCTAACAATGGGTATGAAAGACATAAATG 3' (Sequence ID.3)and
30 the antisense primer was luxrbamh2 5' CGAACTCGAGTCATGATTTTAAAGTATGGGCAA-TCAATTG 3' (Sequence ID.4). The PCR reaction was carried out using *Taq* polymerase (2.5 U) in a reaction

buffer containing 100ng of template DNA, 100 ng of each oligonucleotide, 20 mM TRIS-HCl pH 8.4, 50 mM KCl, 10 mM MgCl₂, 50 mM dNTPs and using hot start conditions followed by 15 cycles of denaturing (94°C for 1 minute), annealing (66°C for 1 min) and synthesis (72°C for 3 min). The fragment was purified and digested using *Bam*HI/*Xho*I and was introduced into pDH51 *Bam*HI/*Sa*I vector to give pDH51luxR (*Xho*I and *Sa*I restriction enzymes produce compatible ends) (Figure 8). The sequence of the insert was determined and compared to the published *LuxR* sequence (Sequence ID. 5) (Devine et al., 1988).

10 Tobacco mesophyll transformation.

Tobacco shoot cultures cv. *Samsun*, were maintained on solidified MS medium + 3% sucrose in a controlled environment room (16 hour day / 8 hour night at 25°C, 55% R.H), were used as the source material for protoplasts. Leaves were sliced parallel to the mid-rib, discarding large veins and the slices were placed in CPW13M (13% mannitol, pH5.6, 860mmol/kg) for 1 hour to pre-plasmolyse the cells. This solution was replaced with enzyme mixture (0.2% cellulase R10, 0.05% macerozyme R10 in CPW9M (CPW13M but 9% mannitol), pH5.6, 600mmol/kg) and incubated in the dark at 25°C overnight (16 hours). The enzyme mixture was passed through a 75µm sieve and the filtrate was centrifuged at 600rpm for 3.5 minutes, discarding the supernatant. The pellet was resuspended in 0.6M sucrose solution and centrifuged at 600rpm for 10 minutes. The protoplasts were removed and diluted with CPW9M (pH5.6, 560mmol/kg) and pelleted by centrifuging at 600rpm for 3.5 minutes. The protoplasts were resuspended in CPW9M, counted, diluted to 2x10⁶/ml in MaMg medium (Negrutui *et al.*, 1987) and aliquoted at 4x10⁵ protoplasts per treatment. 20µg each of effector and reporter plasmid DNA (1mg/ml) were added followed by 200µl PEG solution (Negrutui *et al.*, 1987). The protoplasts were incubated at room temperature for 10 minutes before addition of 5ml MSP19M medium (MS medium, 3% sucrose, 9% mannitol, 2mg/l NAA, 0.5mg/l BAP, pH5.6, 700mmol/kg) in the presence or absence of ligand (N-(-3-oxohexanoyl)-L-homoserine lactone (OHHL)). The protoplasts were cultured in their tubes lying horizontally at 25°C and they were harvested for the GUS assay after 24 hours.

Transient Expression in *Nicotiana plumbaginifolia* suspension cells derived protoplasts

Protoplasts isolation

Protoplasts were isolated from *Nicotiana plumbaginifolia* suspension cells. Suspension cells were sub-cultured once a week in Np suspension medium in 250 ml Erlenmeyer flasks that were shaken at 100 RPM, at 25°C with 16h/8h light/dark regime. Protoplasts were isolated 2 or 3 days after subculture. Two or 2.5 g fresh weight of cells were incubated with 20 ml filter-sterilised enzyme solution and shaken at 40 rpm at 25°C. The enzyme solution comprised 1% cellulysin (Calbiochem 219466), 1 % Macerozyme R10™ (Yakult, Tokyo), and 1% Driselase (Sigma™ D-9515) dissolved Artificial Sea Water Mannitol. After 3 h cell digestion in enzyme solution, they were washed through 100-, 50-um diameter sieves with W5 solution before being collected by centrifugation at 80 x g for 4 min. This method is also used for the isolation of wheat protoplasts.

15

Protoplasts transformation.

Protoplasts were resuspended in W5 medium at densities ranging from 0.1 to 0.2×10^6 /ml and sedimented for 4 min at 80 g. They were then taken up in 0.2 to 0.5 ml of: 0.4 M mannitol, 15 mM MgCl₂, 0.1 % MES, 2 % glucose, pH 5.6 containing 15 µg plasmid DNA. Five minutes later poly-ethylene glycol (PEG) 4000 (Fluka™) at 40% (w/v) in 0.4 M mannitol and 0.1 M Ca(NO₃)₂·4H₂O pH 9.0 was added to give final PEG concentrations of 20%. After half an hour 1 ml 0.2 M CaCl₂·2H₂O were added, and the protoplasts centrifuged for 4 min at 40 g. The protoplasts were then cultured in transformation buffer at $0.1-0.2 \times 10^6$ protoplasts per ml during 48h at 25°C in the dark. This method is also applicable to the transformation of wheat protoplasts.

25

GUS Assays

Transient transformed tobacco protoplasts were harvested by centrifugation at 3000 rpm. by collecting them by centrifugation at 3000 rpm. The supernatant was discarded and the protoplasts resuspended in GUS extraction buffer (Jefferson *et al.*, 1987) for preparation of β-glucoronide extracts. The tubes were vortexed for 1 minute and then spun at 13000rpm for 2 minutes. The supernatant was collected and placed

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in a fresh eppendorf tube. 20µl of the extract were used in the GUS assays. Fluorometric assays for GUS activity were performed with the substrate 4-methylumbelliferyl-D-glucuronide (Sigma™) using a Perkin-Elmer™ LS-35 fluorometer (Jefferson *et al.*, 1987). Protein concentration of tissue homogenates were determined by the Bio-Rad™ protein assay (Bradford, 1976).

Example 2. Transient expression of enhanced LuxR.

The transient expression experiments were carried out as described above. The same reporter plasmid was used but different effector constructs were produced. In order to address whether the presence of a strong activator such as VP16 would enhance transcriptional efficacy of the receptor, it was introduced in both the N- and C- terminal ends of *LuxR*. The VP16 was also fused to the nuclear localisation signal (NLS) from SV40. Both the NLS of SV40 and VP16 were obtained from the yeast two hybrid plasmid pPC86 as a fused fragment which meant that it could be fused as one on to *LuxR*. The N-terminal fusion was constructed by isolating and introducing into pBluescript a *Clal/NotI* fragment of pPC86 containing the SV40 NLS and Gal4 activation domain. The resulting pBluescriptSV40V was digested with *HindIII* and filled in followed by *BglII* digestion. The fragment was introduced into *SmaI/BamHI* pDH51luxR vector (*BamHI* and *BglII* enzymes produce compatible ends) to produce the N-terminal fusion in *LuxR* and yield plasmid pDH51NVLuxR (Figure 10). The methionine start site is provided by the SV40 fragment.

The C-terminal end fusion was carried out by placing at the *RcaI* site in *LuxR* the blunted fragment of NLS/VP16. pDH51LuxR was digested with *RcaI*, blunt ended and ligated to the *Clal/NotI* SV40-Gal4 blunt ended fragment. The fusion resulted in plasmid pDH51LuxRNV which contains four linking amino acids, C A K L, between the end of *LuxR* and the start of the nuclear locating sequence.

The plasmids were tested in tobacco mesophyll protoplasts as described in Example 1, where p221.9lux6 was introduced with or without either of the expression vectors containing the enhanced luxR. GUS assays were carried out as described above.

Example 3. Transient expression of LuxR, LasR and TraR in monocot protoplasts.

Expression plasmid construction.

The vector of choice for expression in monocot transient system was pFun which contains ubiquitin promoter region and the Nos terminator. In between the two there is multiple unique cloning sites. LuxR was transferred into pFun as a BamHI fragment. pLasR was transferred as a BamHI/KpnI fragment. TraR was isolated from *Agrobacterium tumefaciens* by PCR using oligonucleotides TraR1 5' AATTGGTACCCACCATGCAGCACTGGCTGGACAAGTTGACC 3' (Sequence ID. 11) and TraR2 5' AATTGGATCCCAGATCAGCTTTCTTCTGCTTGGCGAGG 3' (Sequence ID 12). The purified fragment was restriction enzyme digested with BamHI and introduced into pFun BamHI vector. On each case the expression vectors were named as follows: pFunLuxR (Figure 13), pFunLasR (Figure 14) and pFunTra (Figure 15).

15

Reporter plasmid construction

Reporter plasmid used with the expression vector encoding for LuxR is the same as that used in the transient experiments carried out in Tobacco (Examples 1 and 2). In the case of reporter vectors for both the expression of LasR and TraR the parental plasmid p221.9 was used. In both cases oligonucleotides encoding the LasBox and the two TraBoxes were synthetically made. The LasBox oligonucleotides are as follows LasBox1 5' TCGACACCTGCGAGTTCTCCGAGGTG 3' (Sequence ID 13) and LasBox2 is 5' TCGACACCTCGGAGAACTCGCAGG TG 3' (Sequence ID 14). The TraBoxes as described by Zhu and Winans, (1999) were used and the oligos are as follows, TraBox1 5' TCGACTACACGTCTAGACGTGTAGG 3' (sequence ID 15), TraBox2 5' TCGACCTACACGTCTAGACGTGTAG 3' (Sequence ID 16) (first pair), TraBox3 5' TCGACTACACGTCTAGACGTGTAAG 3' (Sequence ID 17) and traBox4 5' TCGACTTACACGTCTAGACGTGTAG 3' (second pair) (Sequence ID 18). Equimolar amounts of each pair oligo in the pair were mixed denatured and allow to cool down slowing to form double stranded DNA which had SalI cohesive ends. The double stranded DNA was then ligated into a SalI digested p221.9 vector. Recombinants were screened by colony hybridisation and

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sequenced to ascertain number of elements incorporated into the vector. The vectors were named as follows p221.9Trabox1 (Figure 16), p221.9Trabox2 (Figure 17) and p221.9Lasbox (Figure 11).

5 Example 4. Stable expression of LuxR in Tobacco plants

Binary vector construction

The reporter cassette was isolated from p221.9lux6 by digesting with *EcoRI* and *HindIII* to yield a 2.0kb fragment. The fragment was purified and introduced into pBin 19 *EcoRI/HindIII* vector to produce pBinrepAHL. The three different variants
10 of the *LuxR* receptor, that is *LuxR* and the two enhanced versions were restriction enzyme digested with *EcoRI* (site flanking both sides of the effector cassette) and introduced into a dephosphorylated *EcoRI* pBin19repAHL vector to produce either pAHL1 (*LuxR*) (Figure 18), pAHL2 (SV*LuxR*) (Figure 19) or pAHL3 (*LuxRSV*) (Figure 20).

15

Plant transformation

The plant transformation construct pAHL1 (Figure 18), pAHL2 (Figure 19) and pAHL3 (Figure 20), containing *LuxR* or chimeric *LuxR* and a reporter gene cassette, were transformed into *Agrobacterium tumefaciens* LBA4404 using the
20 freeze/thaw method described by Holsters *et al.* (1978). Tobacco (*Nicotiana tabacum* cv Samsun) transformants were produced by the leaf disc method (Horsch *et al.*, 1988). Shoots were regenerated on medium containing 100mg/l kanamycin. After rooting, plantlets were transferred to the glasshouse and grown under 16 h light/ 8 h dark conditions

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PCR analysis

Analysis of transgenic tobacco plants by PCR was carried out using leaf sample extracted in 300µl of extraction buffer. The DNA was precipitated with isopropanol at 4°C for 10 minutes and then centrifuged. The pellet was dried and resuspended in
30 100µl of TE (10mM Tris HCl pH 8.0, 1mM EDTA). 2.5 µl were placed in a 500 µl eppendorf tube and a master mix containing buffer, dNTPs and oligonucleotides was

added. The *Taq* polymerase (Gibco-BRL™) was added after samples were denatured for 3 minutes.

Chemical treatments

5 OHHL was dissolved in methanol (Sigma) and the stock maintained at -20°C. The compound was diluted in growth media used to germinate seedlings. Uninduced seedlings were treated with equivalent amount of methanol. Seeds were germinated in MS media supplemented with 0.8% (w/v) agar. Seedlings were collected 2 days post-germination (two cotyledon stage).

10

GUS Assay

In seedling induction experiments 10 two day old seedlings were collected and flash frozen in liquid nitrogen. The seedlings were homogenised in 300 µl of GUS extraction buffer and centrifuged for 5 minutes at 13000rpm. The supernatant was
15 used for both GUS and Bio-Rad™ protein assays.

Example 5 Stable constitutive expression of *yenI* gene in tobacco plants

Binary vector construction.

pBDHELI (Figure 21) was constructed by fusing the alfalfa mosaic virus
20 (AMV) translation enhancer sequence from pBi526 (Datla et al., Plant Science 94, 139-140 (1993)) to the *yenI* coding sequence from *Yersinia enterocolitica*. AMV-*LuxI* gene fusion was directional cloned into pDH51 (Pietrzak et al., 1986) vector to produce pDHELI. A 1.8kb fragment of pDHELI was cloned into pBin19 (Bevan, 1994) to give pBDHELI (Figure 21). The plasmid was introduced into *Agrobacterium*
25 as described above. A tobacco plant population of was produce and screened as detailed above. A high expressor of *yenI* gene was selected by their ability to synthesised OHHL. The assay consists of placing a leaf of the transgenic plant on an agar plate overnight. The leaf was then removed and the *cviI* mutant of *Chromobacterium violaceum* spread over the plate. Violacein (a purple pigment)
30 production by the bacterium can be seen when OHHL had diffused out of the leaf and into the agar (Figure 24).

Example 6. LuxR is activated by compounds produced by *yenI* plants.

The root of a transgenic tobacco plant containing the *yenI* expression cassette, that was grown in tissue culture under aseptic conditions, was placed on an LB agar plate and overlain with top agar containing *E. coli* with the plasmid pSB401 (Figure 22). This strain expresses the *lux* operon (i.e. *luxR*, *lux* ABCDE) and will bioluminesce in response to OHHL and HHL. Figure 24 shows that acyl-homoserine lactones produced by *yenI* introduction into tobacco plants are capable of activating *LuxR* harboured in *E. coli* and activate reporter gene expression. These data suggest that introduction of *LuxR* expression and reporter cassettes into plants harbouring the *yenI* gene will result in activation of reporter gene expression.

Example 7. Cross of *LuxR* tobacco high expressor plant with *yenI* tobacco plant.

Plants are produced by crossing the *yenI* expressing plant with the AHL switch plants. The seed of the progeny was collected and assayed for GUS activity as described above. GUS activity was assayed for in all tissues and different ages as the expectation was that GUS protein would be present in all tissues expressing the *yenI* gene. *yenI* is under control of the 35S CaMV promoter as is the *LuxR* protein in the AHL switch. Initially, progeny seedlings were grown in 1/2MS and were collected 2 days post-germination or when cotyledons were fully extended.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

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CLAIMS

1. A method of initiating transcription of a target gene in a eukaryotic cell comprising:
 - 5 (a) providing a eukaryotic cell which is capable of producing a response protein;
 - (b) inserting into the genome of said cell a polynucleotide defining an inducible promoter sequence operably linked to and capable when induced of initiating transcription of said target gene; and
 - 10 (c) applying to said cell a chemical inducer capable of binding to said response protein whereby said chemical inducer binds to said response protein to form an inducing complex which binds to and induces said inducible promoter thereby initiating transcription of said target gene.
- 15 2. A method according to claim 1 wherein a polynucleotide is inserted into the genome of said eukaryotic cell which polynucleotide provides for the production of the said response protein.
3. A method according to claim 1 or 2 wherein the inducible promoter comprises
20 the nucleotide sequence depicted as SEQ ID No. 2 and the response protein comprises the amino acid sequence depicted in SEQ ID No. 16.
4. A method according to claim 3 wherein the said inducible promoter comprises a polynucleotide which is the complement of one which binds to SEQ ID No.
25 2 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein the said polynucleotide is still capable of acting as an inducible promoter upon binding with the said inducing complex.
30
5. A method according to claim 3 or 4 wherein the polynucleotide encoding the said response protein comprises the sequence depicted in SEQ ID No. 5.

6. A method according to claim 5 wherein the polynucleotide encoding the said response protein comprises the complement of one which binds to SEQ ID No. 5 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein the said polynucleotide still encodes a protein which is capable of forming an inducing complex with the said chemical inducer.
- 10 7. A method according to any one of claims 2 to 6 wherein the polynucleotide encoding the said inducible promoter contains the region depicted as SEQ ID No. 21.
- 15 8. A method according to any one of claims 1 to 7 wherein the said eukaryotic cell is a plant cell.
9. A method according to claim 8 wherein the plant cell is from a plant selected from the group consisting of: melons, mangoes, soybean, cotton, tobacco, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tomato, alfalfa, lettuce, 20 maize, wheat, sorghum, rye, bananas, barley, oat, turf grass, forage grass, sugar cane, pea, field bean, rice, pine, poplar, apple, peaches, grape, strawberries, carrot, lettuce, cabbage, onion, citrus or nut plants.
10. A method according to any one of claims 1 to 9 wherein the said chemical 25 inducer is N-(3-oxo)hexanoyl-L-homoserine lactone or a functional equivalent thereof.
11. A method according to any one of claims 8 to 10 wherein the said chemical 30 inducer is applied to the plant cell as part of an agriculturally acceptable formulation.

12. A method according to any one of claims 2 to 11 wherein the polynucleotide encoding the said response protein is bounded by a promoter and a terminator sequence.
- 5 13. A method according to claim 12 wherein the promoter is inducible.
14. A method according to claim 13 wherein the promoter is selected from the group consisting of: Alc A/ R switch system, the GST switch system and the ecdysone switch.
- 10 15. A method according to claim 12 wherein the promoter is constitutive or developmentally regulated.
16. A method according to claim 15 wherein the promoter is selected from the group consisting of: Cauliflower mosaic virus 35S/19S, maize Ubiquitin and *Arabidopsis* Ubiquitin 3.
- 15 17. A method according to any one of claims 1 to 16 wherein the said target gene encodes β -glucuronidase; *Bacillus thuringensis* toxin; barnase or barstar.
- 20 18. A method of providing plants containing an inducible target gene comprising:
- (a) inserting into a plant cell which cell provides for production of a response protein a polynucleotide defining an inducible promoter sequence operably linked to said target gene; and
- 25 (b) regenerating morphologically normal fertile plants thereof; and
- (c) applying to the population of regenerants a chemical inducer or a functional variant thereof capable of binding to said response protein, whereby said chemical inducer binds to said response protein to form an inducing complex which binds to and induces said inducible promoter thereby initiating
- 30 transcription of said target gene; and
- (d) selecting those plants which are expressing the said target gene.

19. A method according to claim 18 wherein the said inducible promoter sequence comprises the nucleotide sequence depicted as SEQ ID No. 2 or a functional variant thereof and the response protein comprises the amino acid sequence depicted as SEQ ID No 16 or a functional variant thereof and the said
5 chemical inducer is N-(3-oxo)hexanoyl-L-homoserine lactone or a functional equivalent thereof.
20. A method according to claim 18 wherein the said inducible promoter sequence contains the nucleotide sequence depicted as SEQ ID No. 10 or a functional
10 variant thereof and the response protein comprises the amino acid sequence depicted as SEQ ID No 17 or a functional variant thereof and the said chemical inducer is N-(3-oxo)dodecanoyl-L-homoserine lactone or a functional equivalent thereof.
- 15 21. A method according to claim 18 wherein the said inducible promoter sequence contains the nucleotide sequence depicted as SEQ ID No. 12 or a functional variant thereof and the response protein comprises the amino acid sequence depicted as SEQ ID No 18 or a functional variant thereof and the said
20 chemical inducer is N-(3-oxo)octanoyl-L-homoserine lactone or a functional equivalent thereof.
22. Plants produced according to a method of any one of claims 18 to 21.
23. Plants according to claim 22 which plants are selected from the group
25 consisting of: melons, mangoes, soybean, cotton, tobacco, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tomato, alfalfa, lettuce, maize, wheat, sorghum, rye, bananas, barley, oat, turf grass, forage grass, sugar cane, pea, field bean, rice, pine, poplar, apple, peaches, grape, strawberries, carrot, lettuce, cabbage, onion, citrus or nut plants.
- 30 24. A DNA construct which comprises a first polynucleotide region which comprises an inducible promoter sequence operably linked to and capable when

induced of initiating transcription of a target gene and a second polynucleotide region which provides for the production of a response protein wherein upon contact of the said response protein with a chemical inducer the said response protein binds the chemical inducer to produce an inducer complex which binds the said inducible promoter sequence thereby initiating transcription of the said target gene.

25. A DNA construct according to claim 24 wherein the said first polynucleotide comprises the nucleotide sequence depicted as SEQ ID No. 2 or a functional variant thereof and the second polynucleotide region comprises a polynucleotide which encodes the amino acid sequence depicted as SEQ ID No. 16 or a functional variant thereof.

26. A DNA construct according to claim 24 wherein the said first polynucleotide comprises the nucleotide sequence depicted as SEQ ID No. 10 or a functional variant thereof and the second polynucleotide region comprises a polynucleotide which encodes the amino acid sequence depicted as SEQ ID No. 17 or a functional variant thereof.

27. A DNA construct according to claim 24 wherein the said first polynucleotide comprises the nucleotide sequence depicted as SEQ ID No. 12 or a functional variant thereof and the second polynucleotide region comprises a polynucleotide which encodes the amino acid sequence depicted as SEQ ID No. 18 or a functional variant thereof.

28. A DNA construct according to any one of claims 24 to 27 wherein the said second polynucleotide region comprises a promoter operably linked to the polynucleotide encoding the said response protein.

29. A DNA construct according to claim 28 wherein the said promoter is inducible.

30. A DNA construct according to claim 28 wherein the said promoter is constitutive or developmentally controlled.
31. Use of a DNA construct according to any one of claims 24 to 30 in the
5 production of a plant containing a target gene of which the expression may be controlled by the application of a chemical inducer to the said plant.
32. A method of screening compounds in a bioassay comprising applying an amount of a chemical to a plant which plant contains a polynucleotide
10 comprising an inducible promoter operably linked to a region encoding a reporter protein and which plant is also capable of producing a response protein and testing the said plant for the production of the said reporter protein.
33. A method of selectively controlling pests in a field which field comprises crop
15 plants and pests wherein the plants are those obtained according to claim 18 and the said target gene encodes a target protein which is capable of controlling the said pests said method comprising applying to the plants an amount of a chemical inducer which is sufficient to bind to the said response protein to produce the said inducing complex which is capable of initiating
20 transcription of the target gene which provides for the production of the target protein in an amount which is sufficient to control the said pests.
34. A method of providing a plant which contains a target gene which is inducibly controlled comprising:
25 (a) inserting into a first plant cell a polynucleotide comprising a first inducible promoter operably linked to a target gene and regenerating a first morphologically normal fertile plant therefrom;
(b) inserting into a second plant cell a polynucleotide comprising a promoter operably linked to a region encoding a response protein which is
30 capable of binding to a chemical inducer to produce an inducing complex which is then capable of binding the said inducible promoter to allow for the

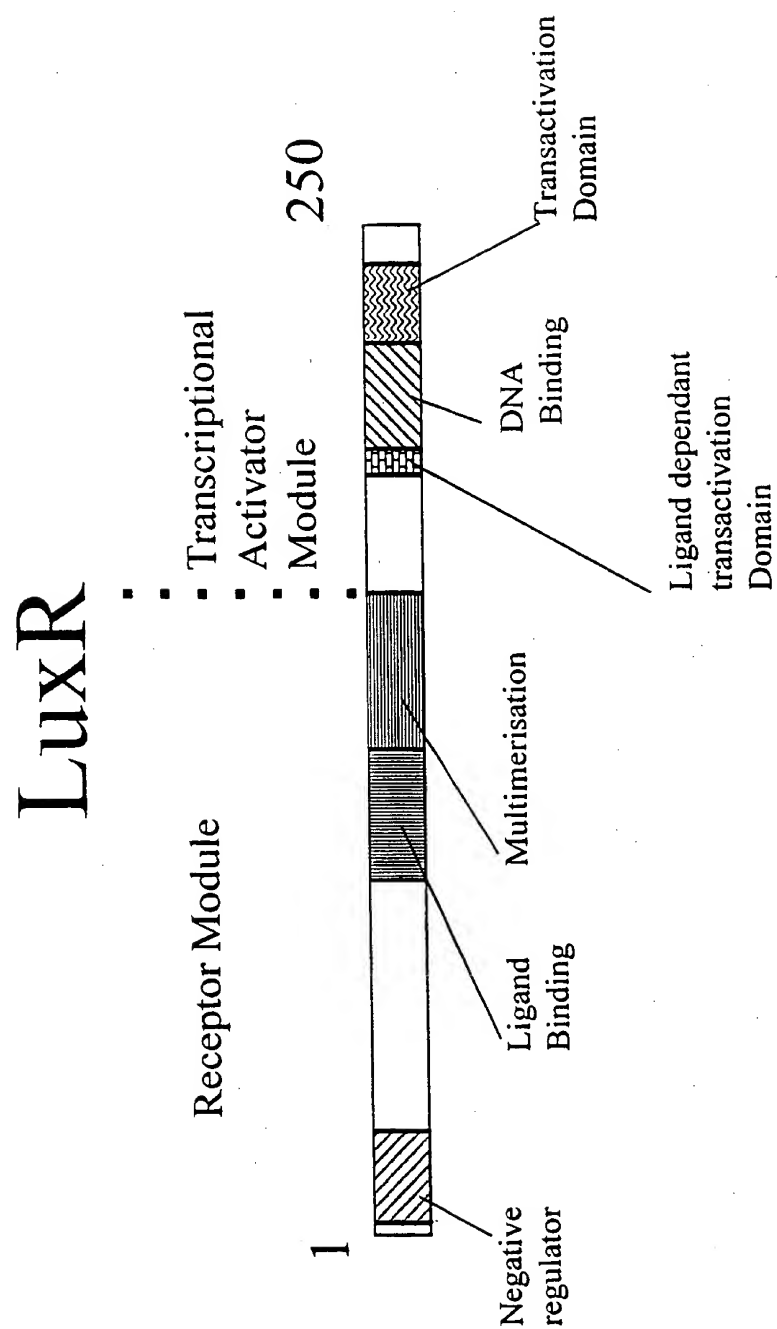
initiation of transcription of the said target gene and regenerating a second morphologically normal fertile plant therefrom;

(c) cross pollinating said first plant with the said second plant or said second plant with said first plant and harvesting the seed therefrom; and

5 (d) growing said seed and applying to the resultant plants an amount of said chemical inducer which provides an inducing complex capable of binding the said inducible promoter to allow for the initiation of transcription of the said target gene.

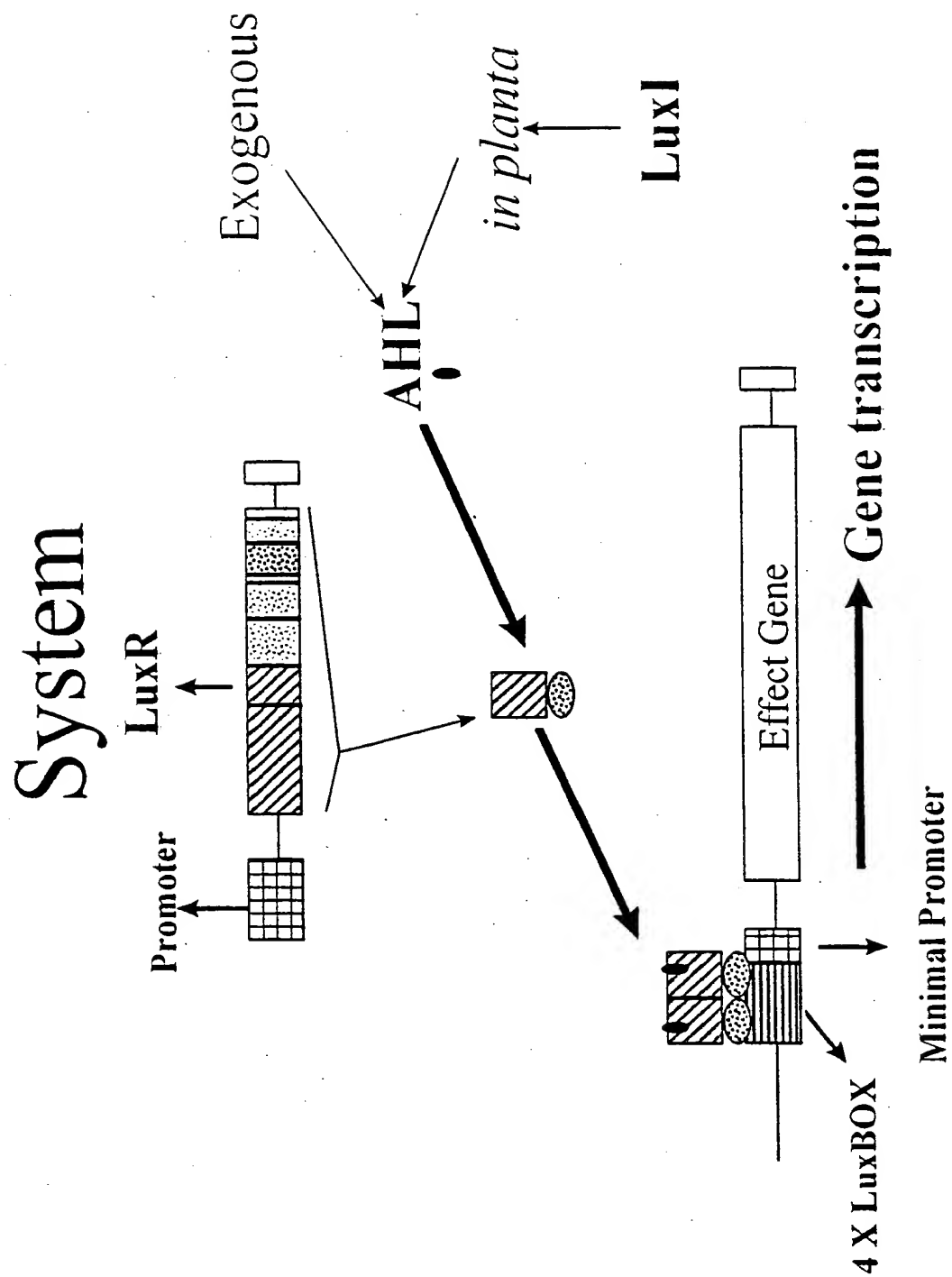
1/24

FIGURE 1



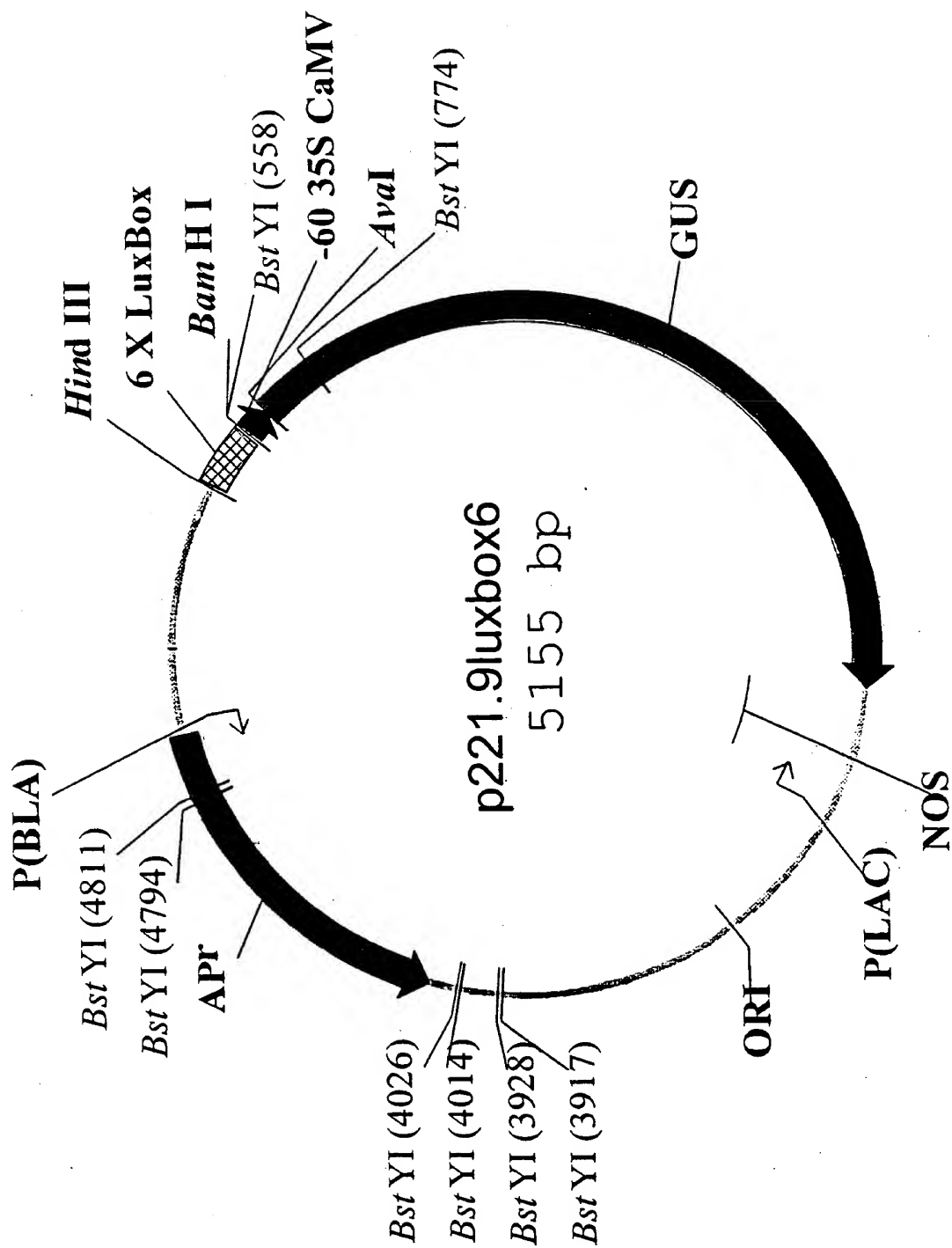
2/24

FIGURE 2



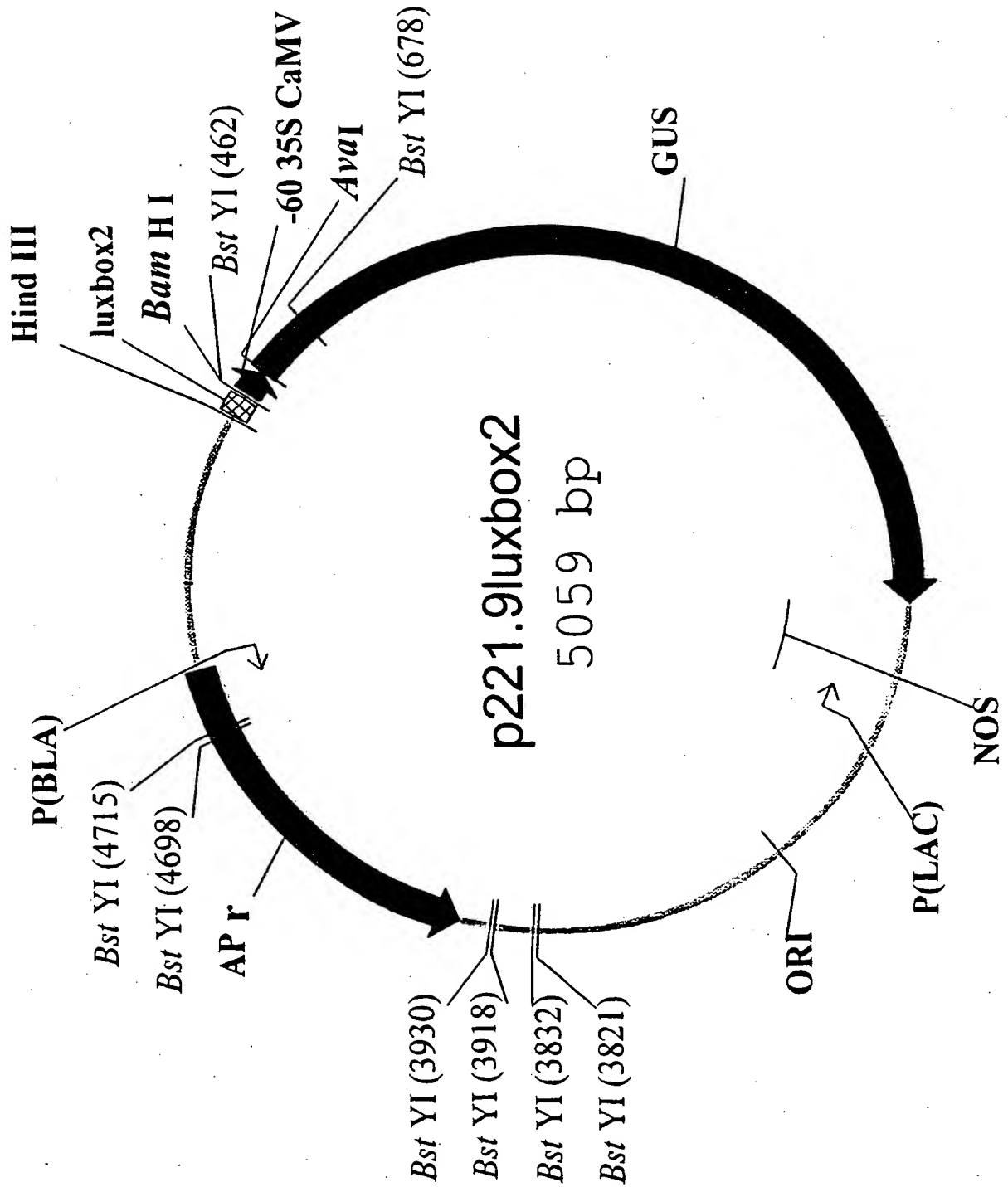
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FIGURE 3



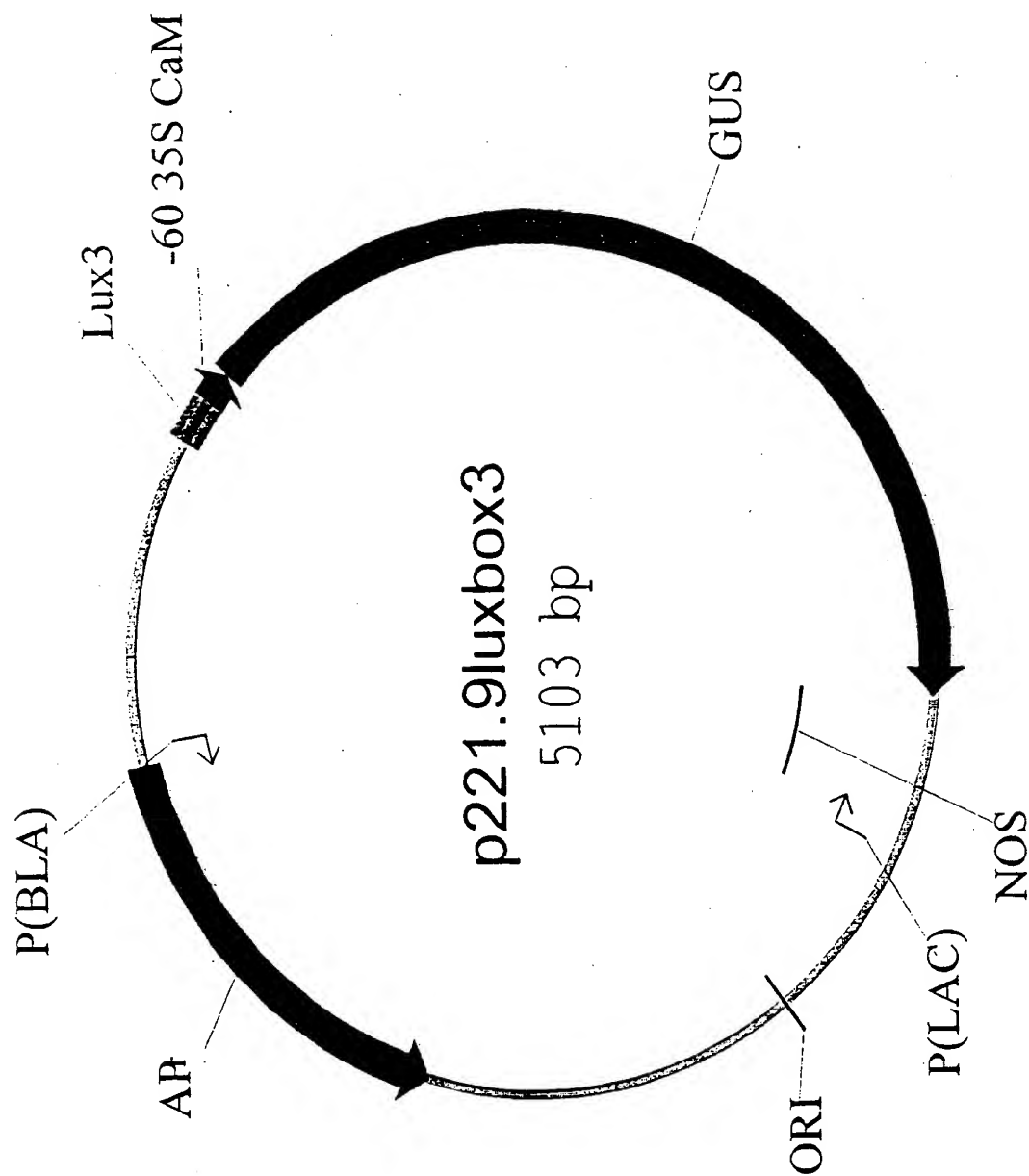
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FIGURE 4



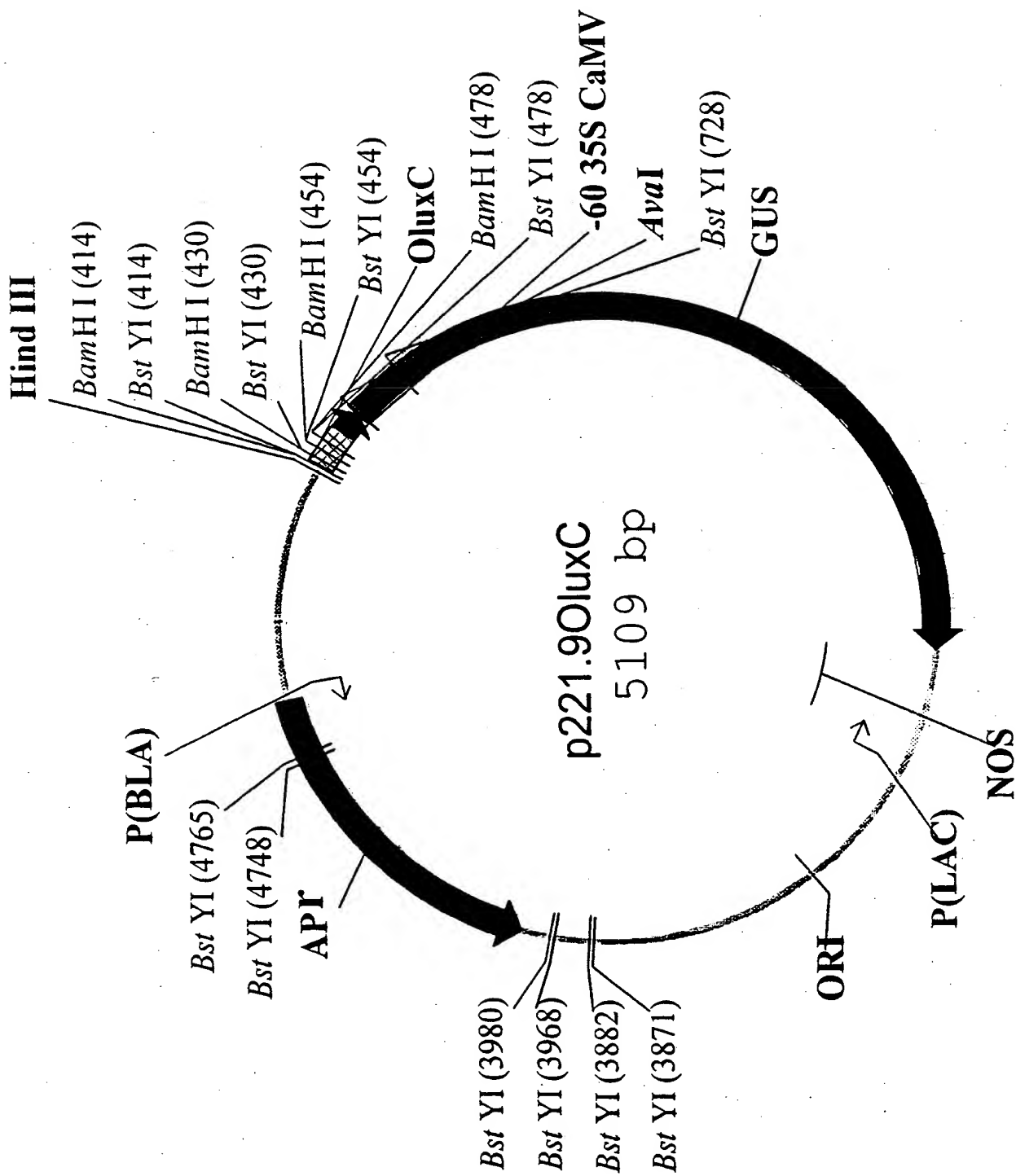
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FIGURE 5



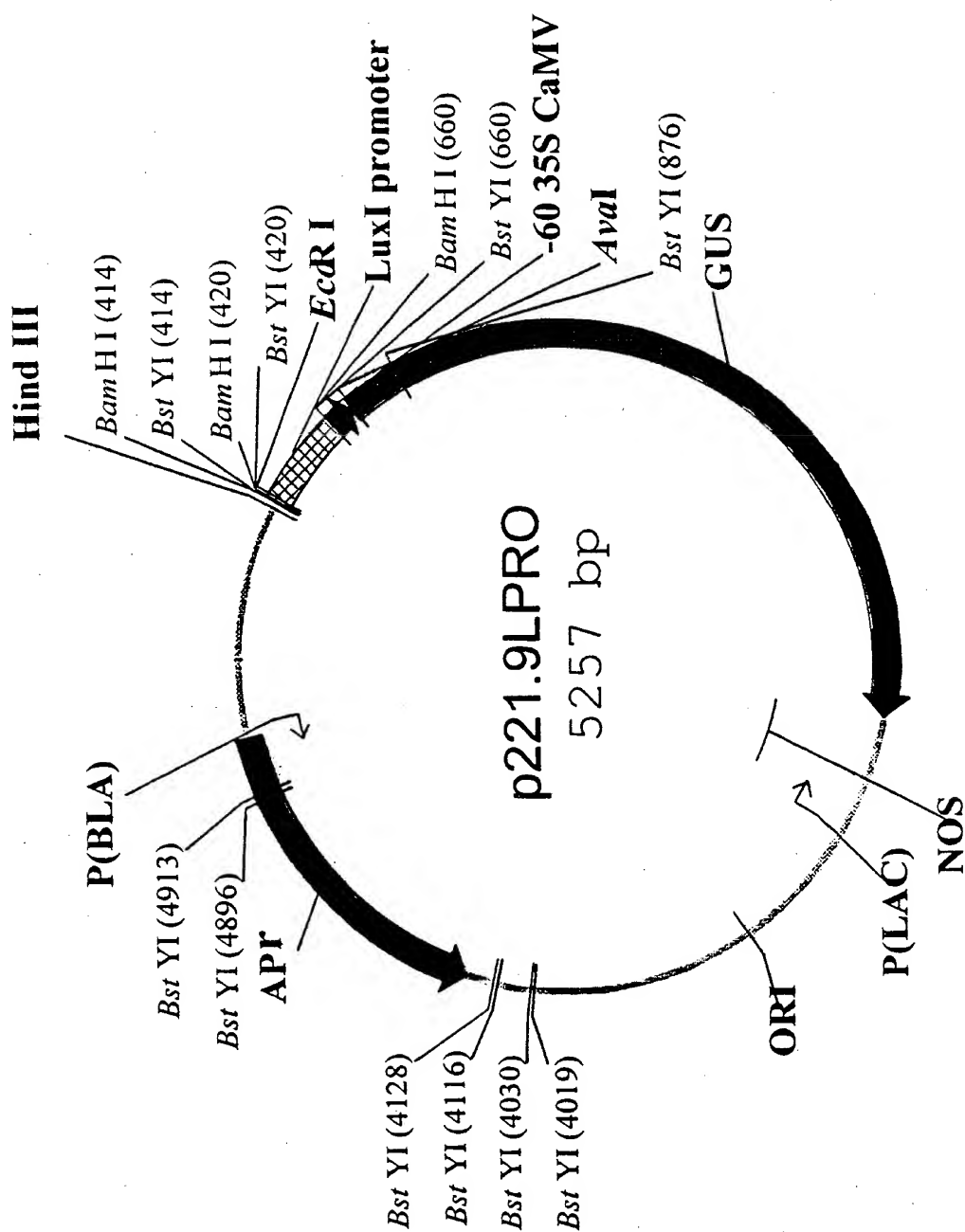
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FIGURE 6



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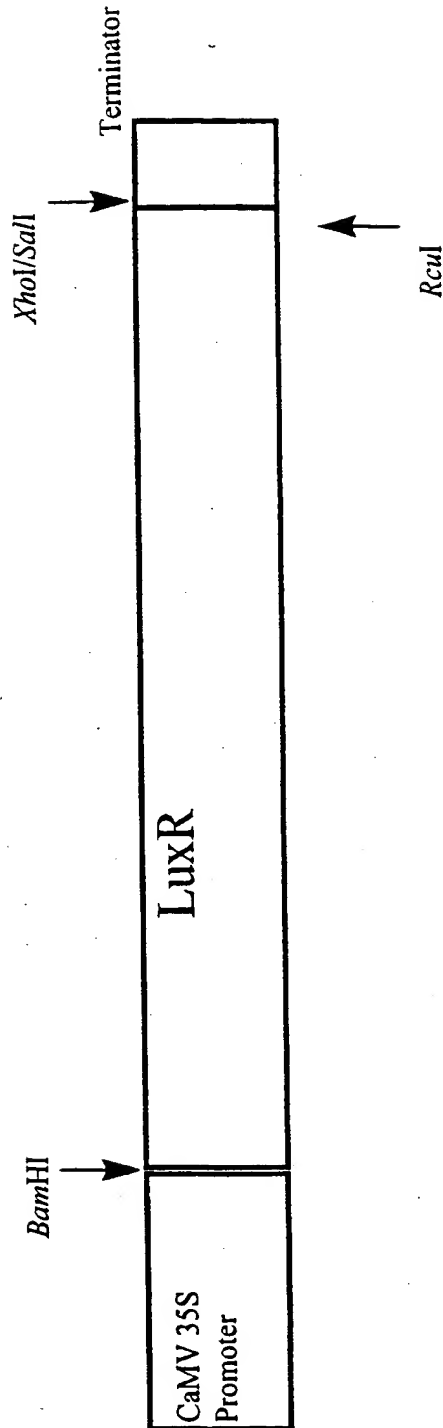
FIGURE 7



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FIGURE 8

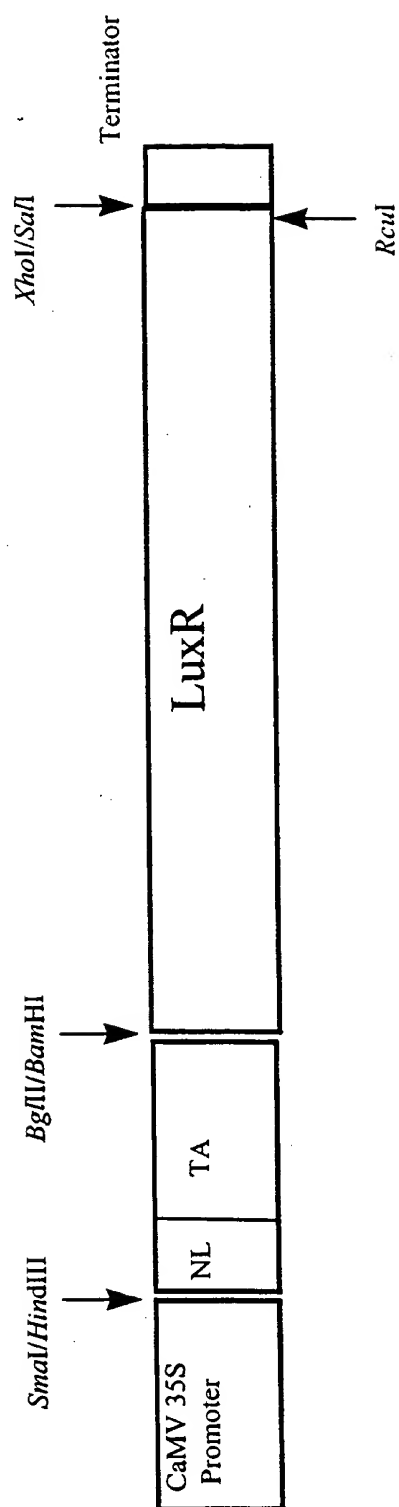
Expression cassette containing LuxR



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FIGURE 9

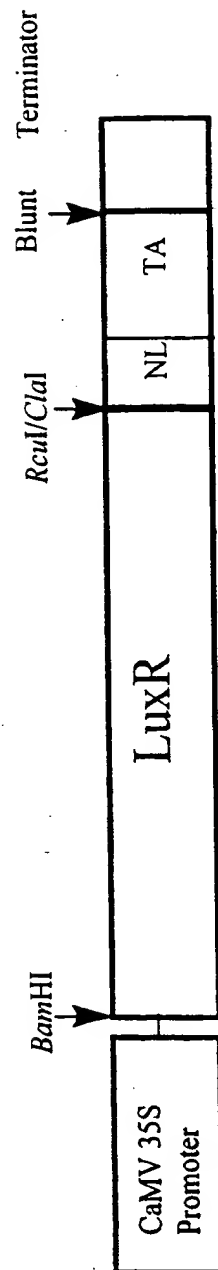
Expression cassette containing NVLuxR



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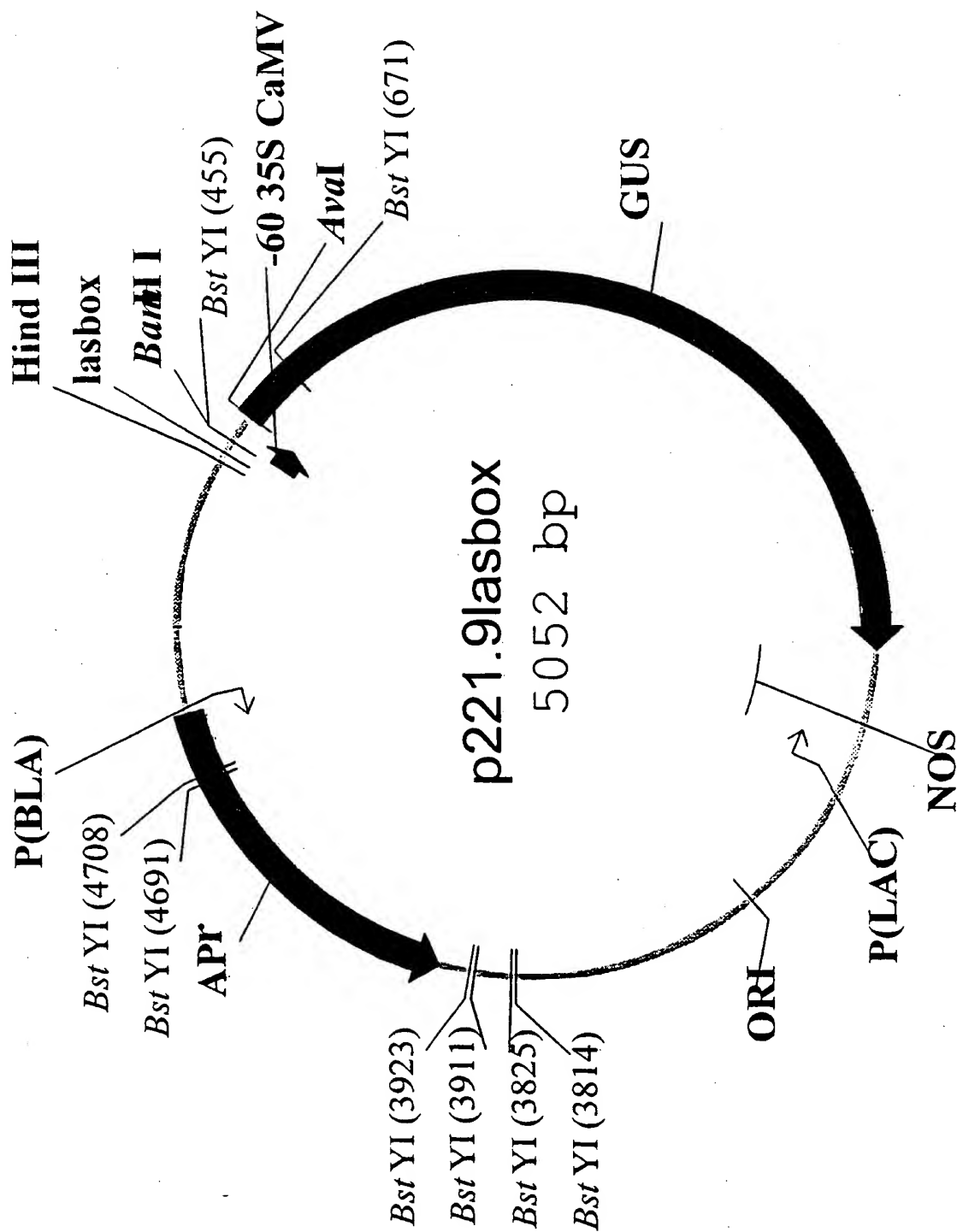
FIGURE 10

Expression cassette containing LuxNV



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FIGURE 11



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FIGURE 12

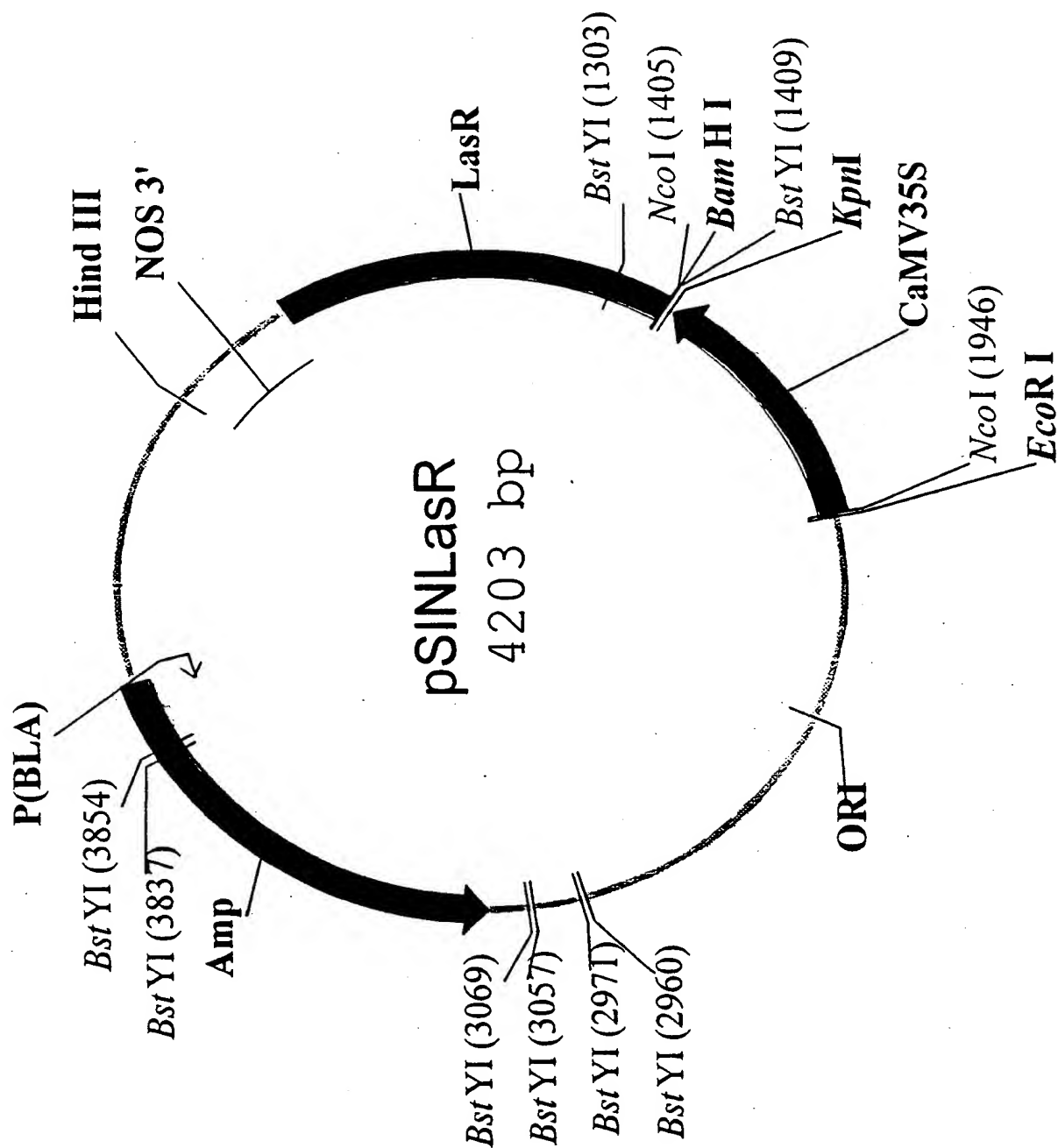


FIGURE 13

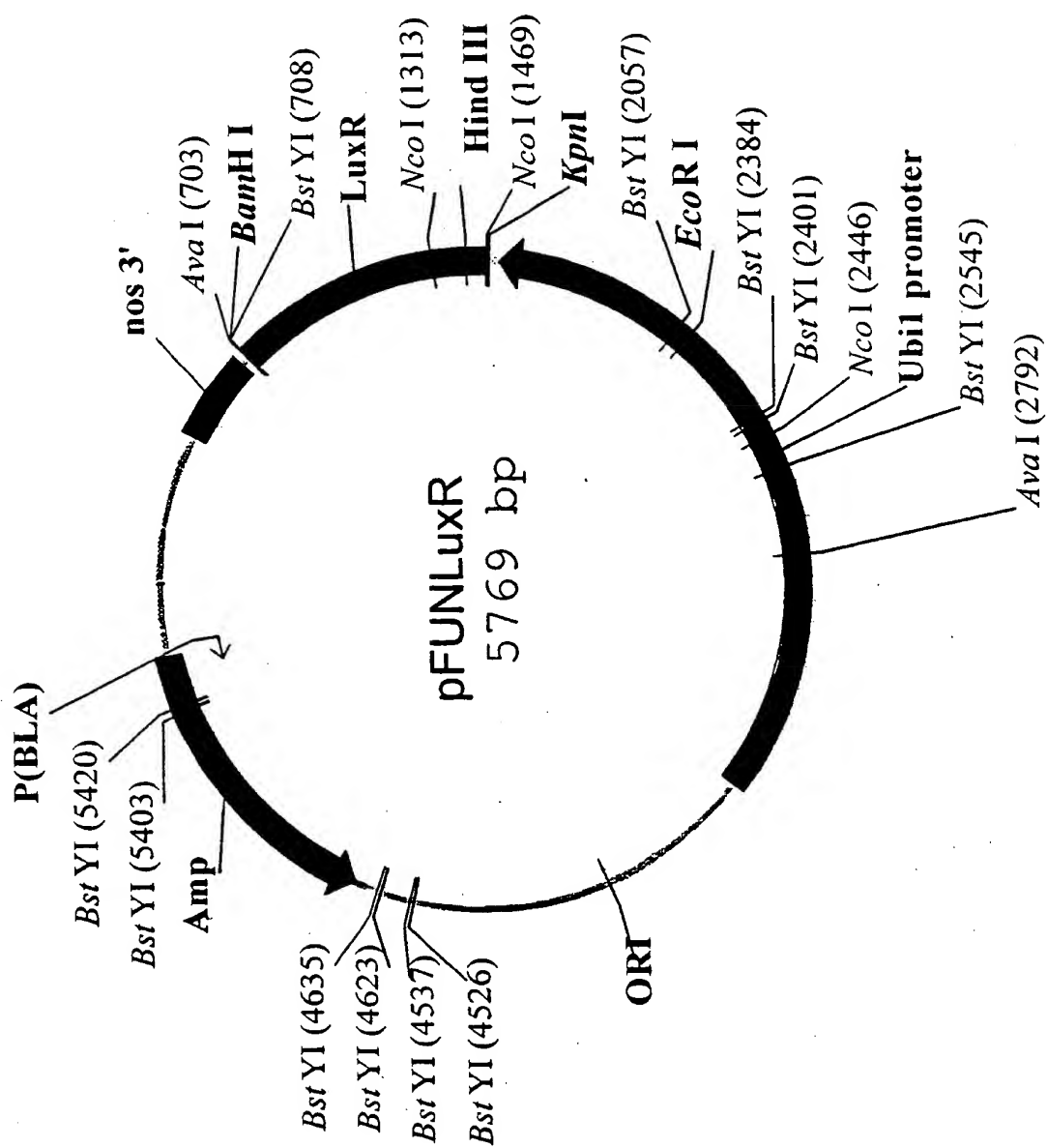


FIGURE 14

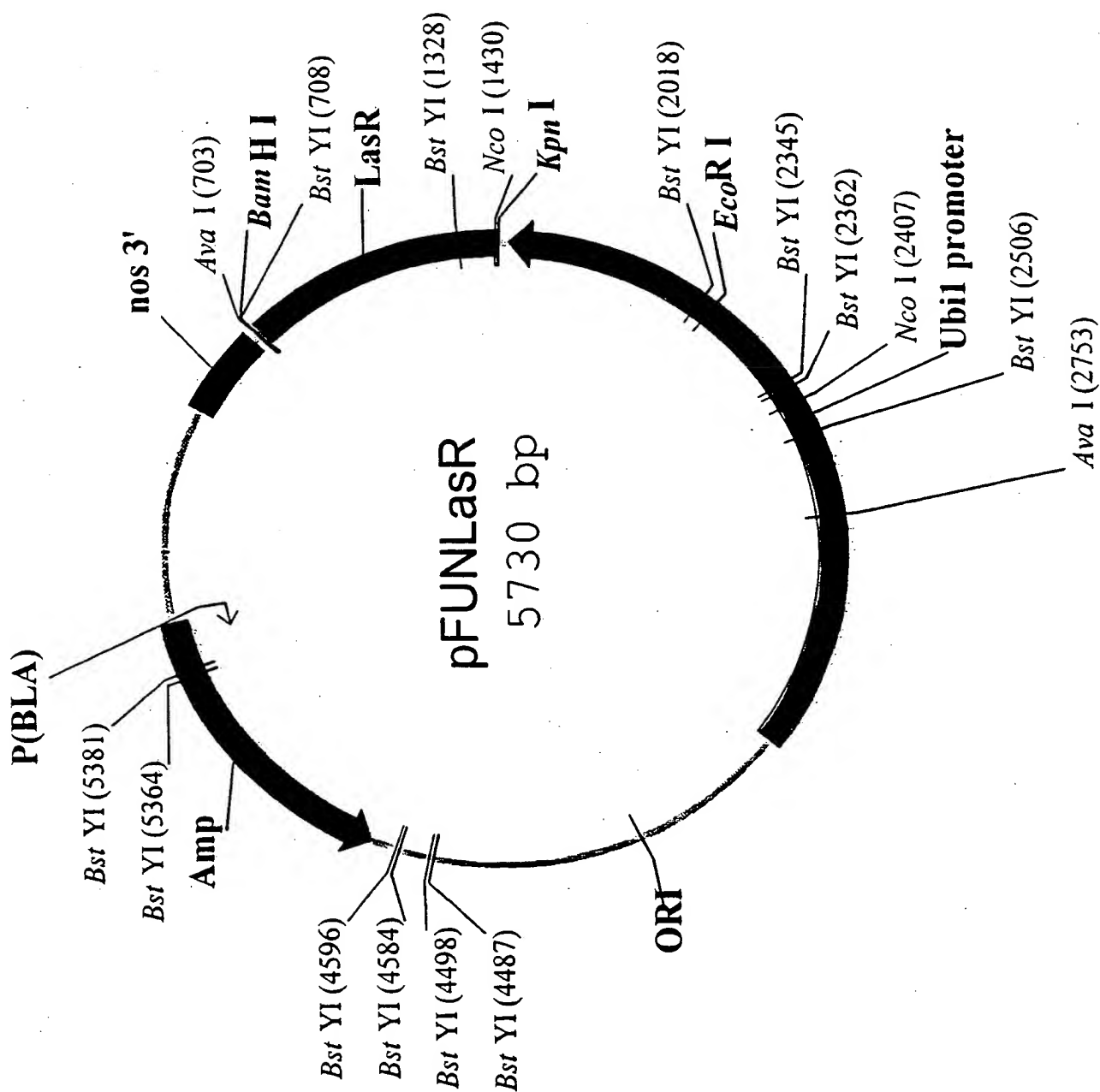


FIGURE 15

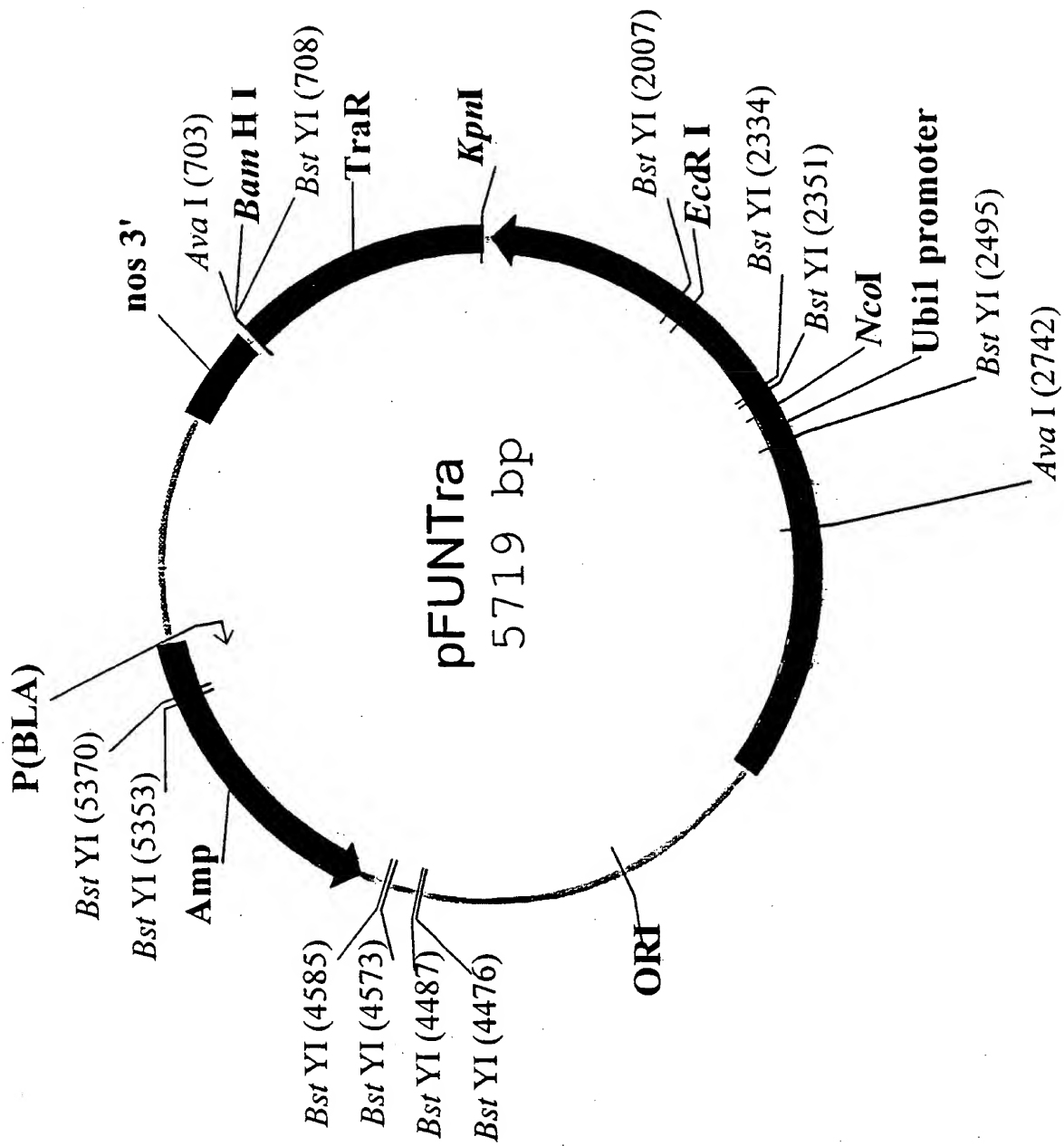
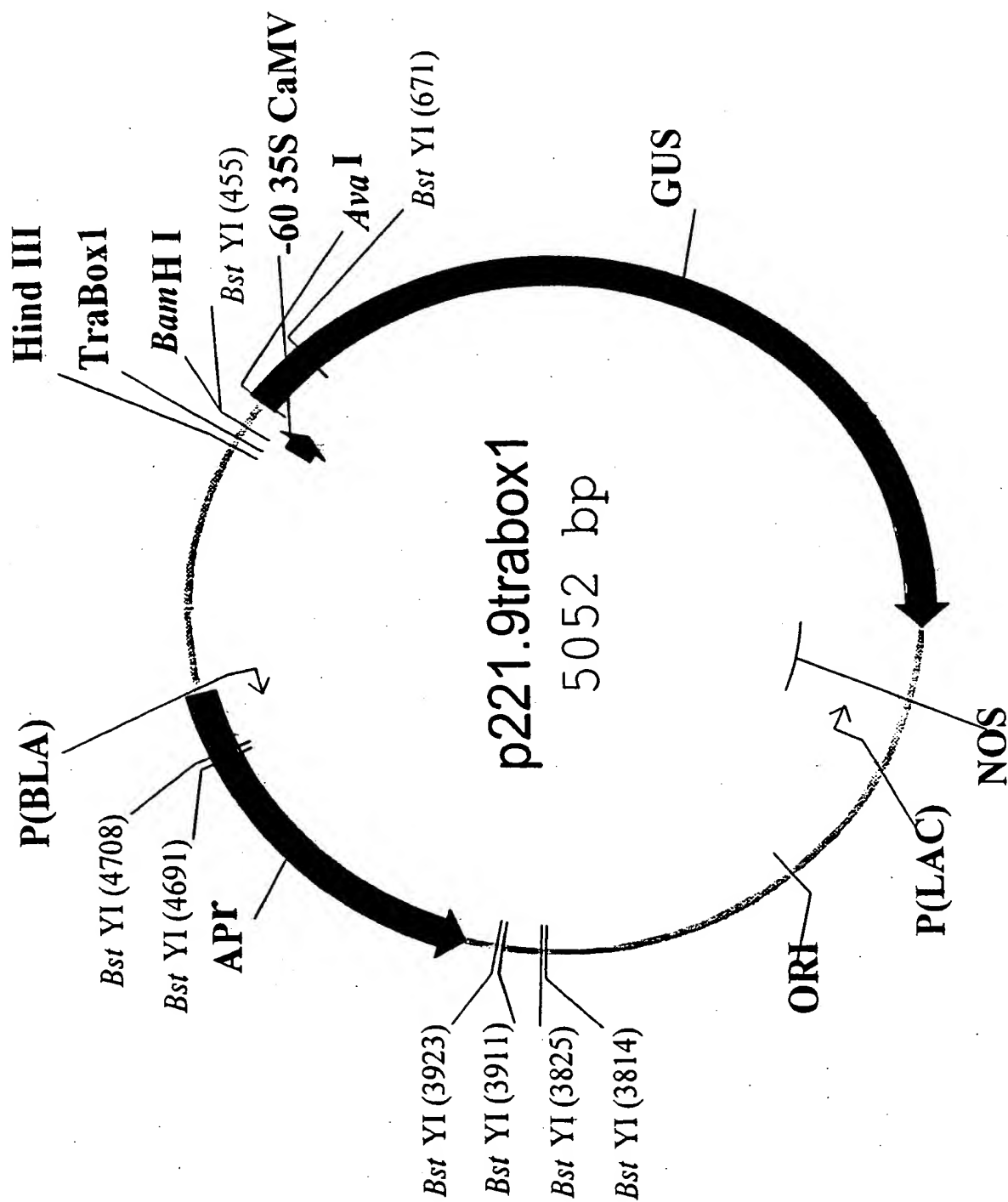


FIGURE 16



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FIGURE 17

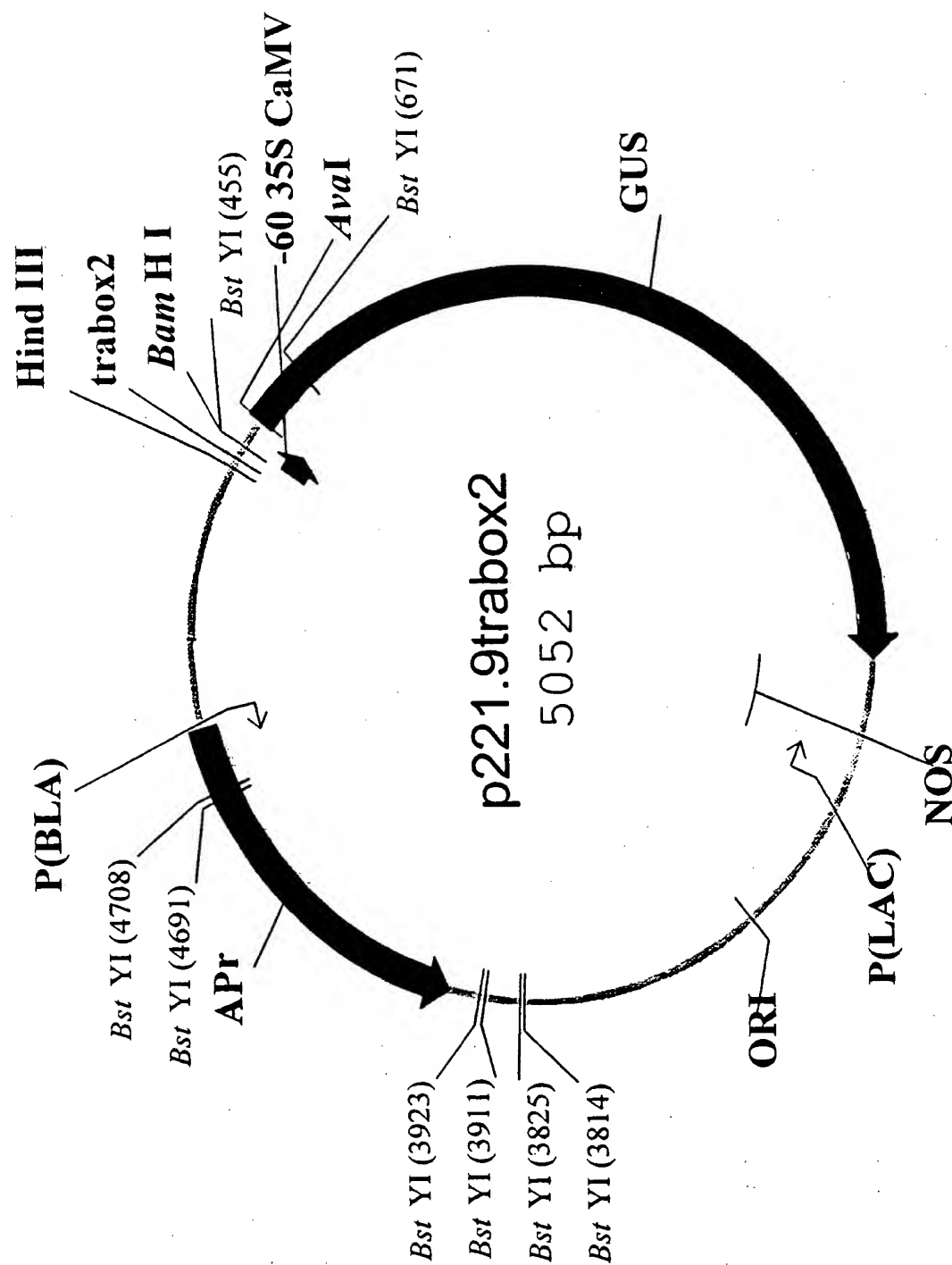


FIGURE 18

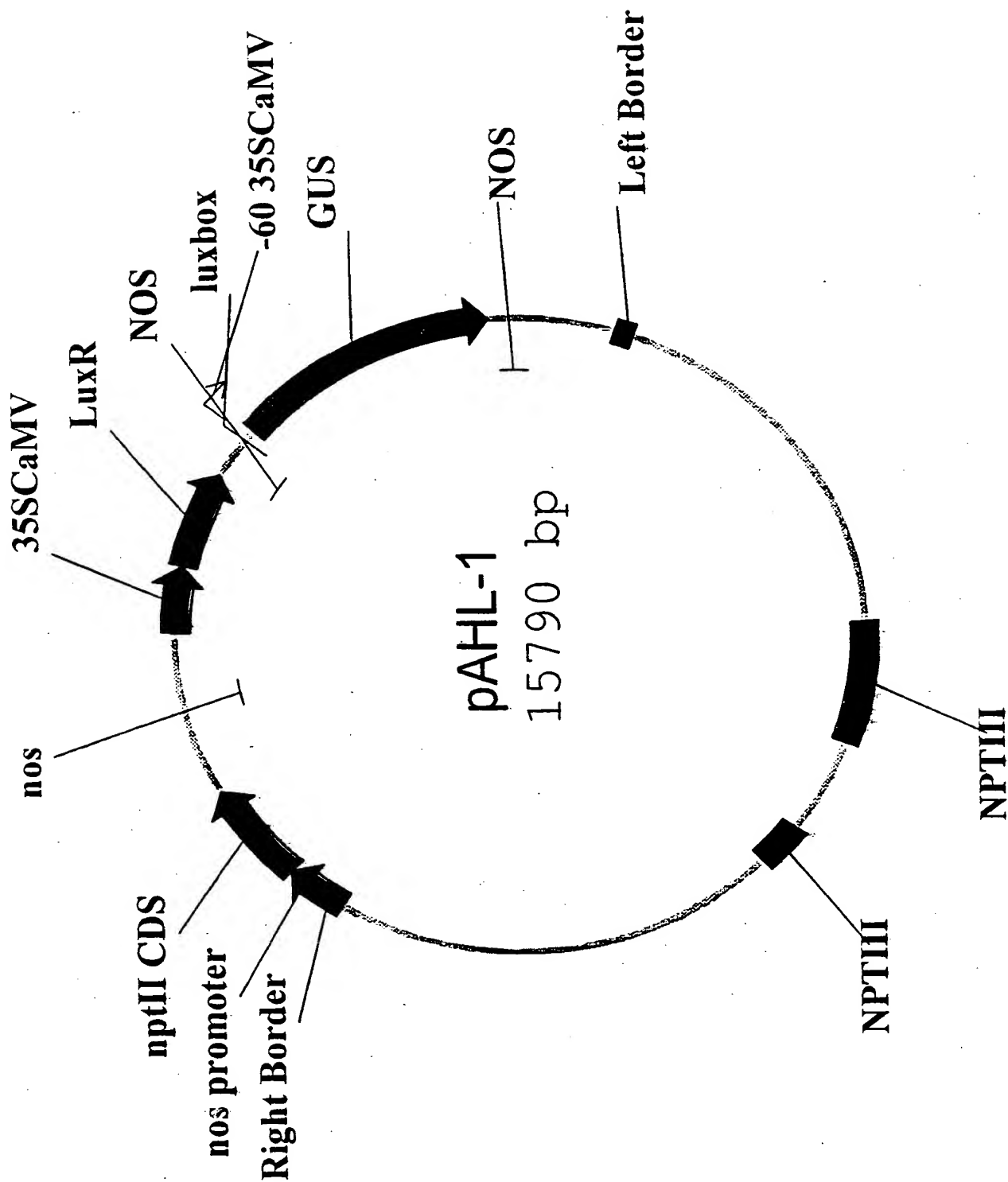


FIGURE 19

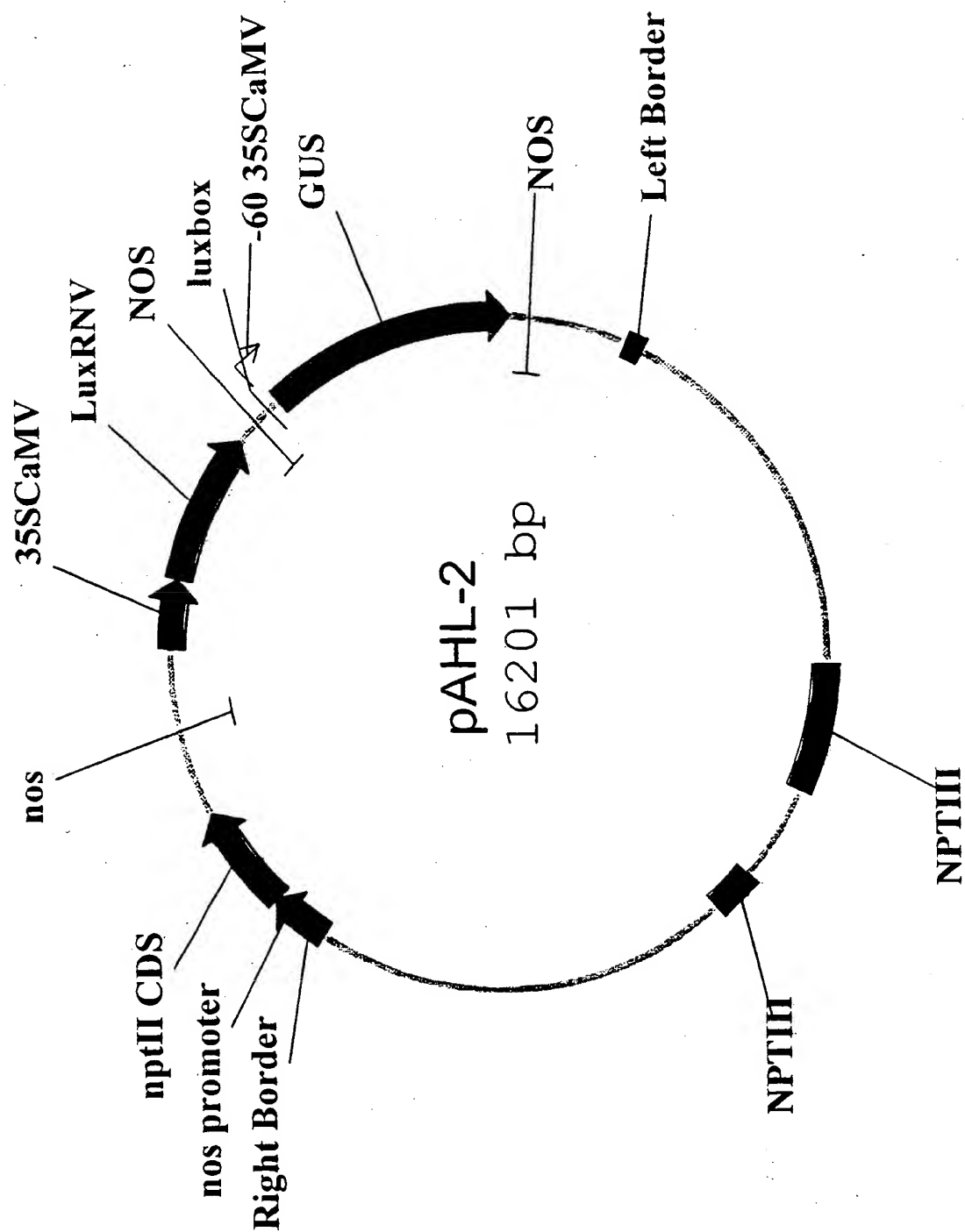


FIGURE 20

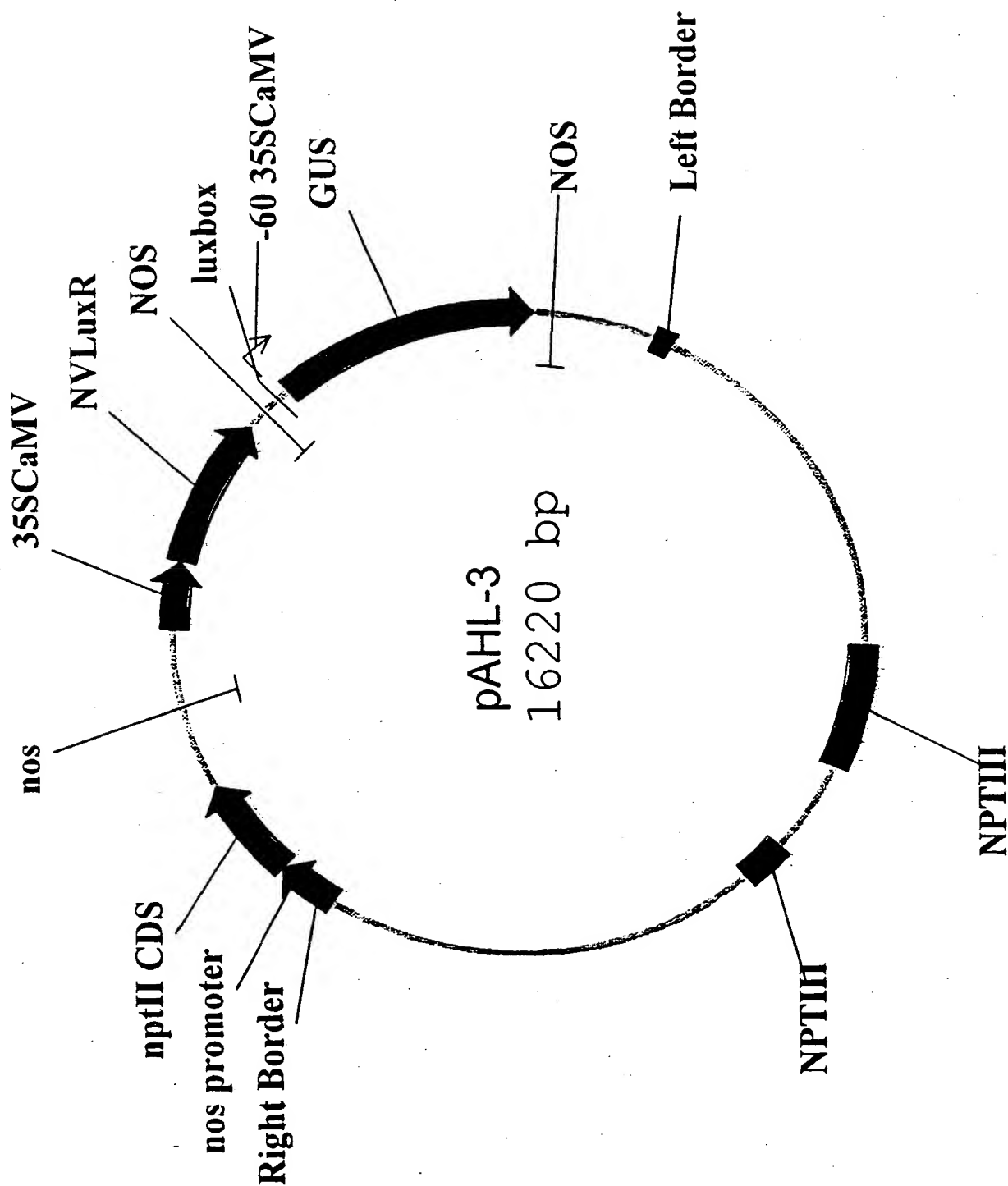


FIGURE 21

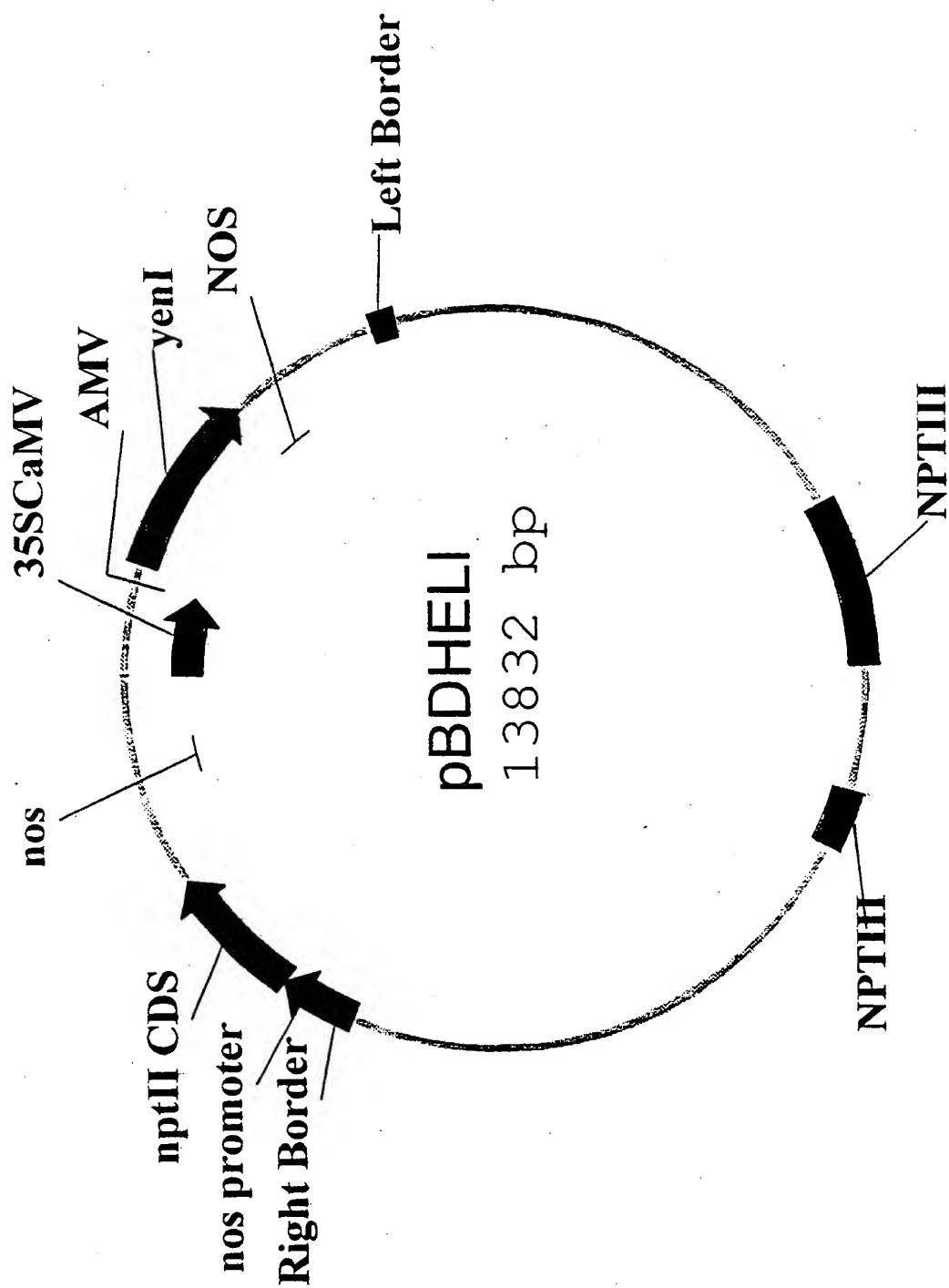


FIGURE 22

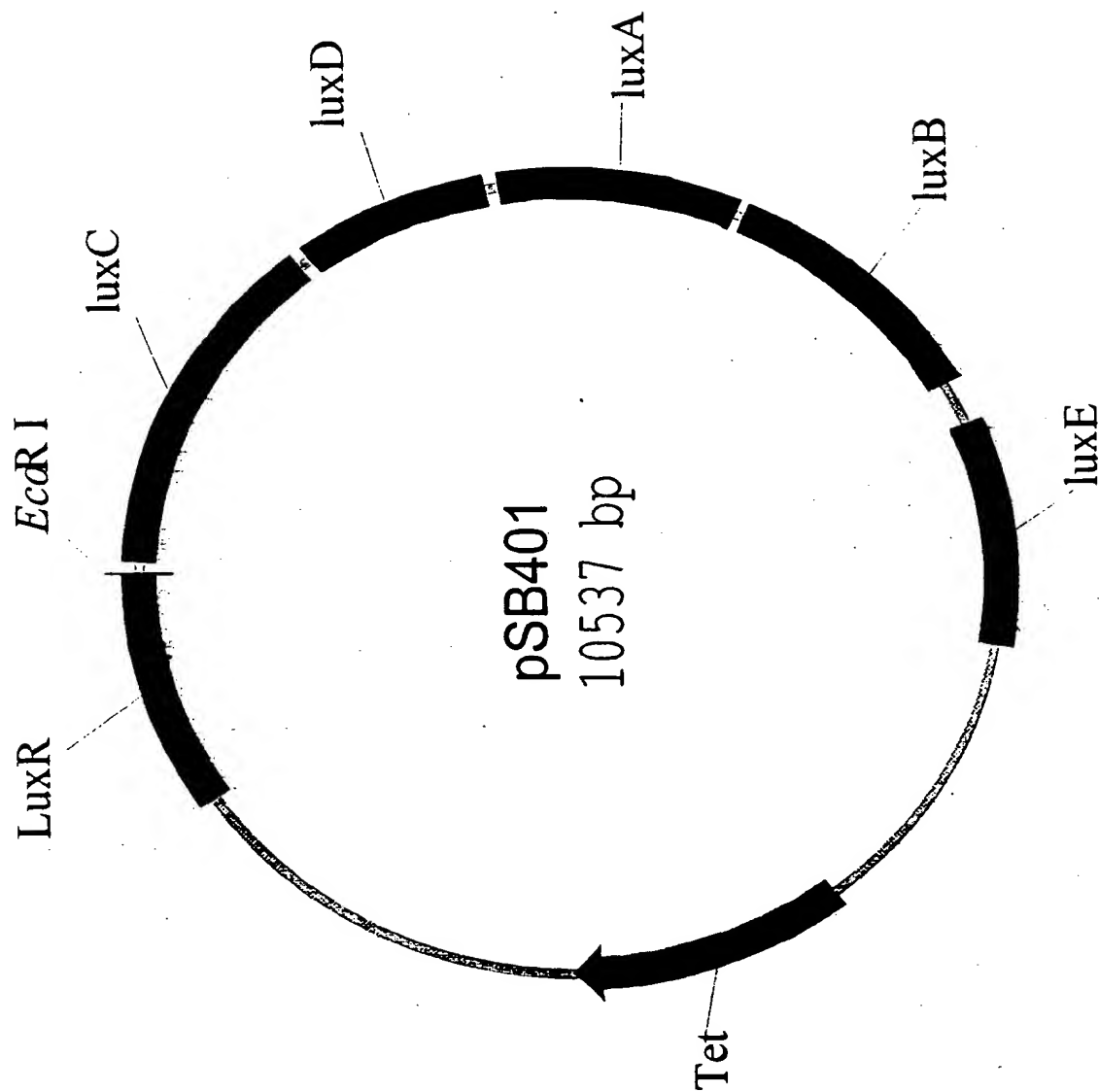
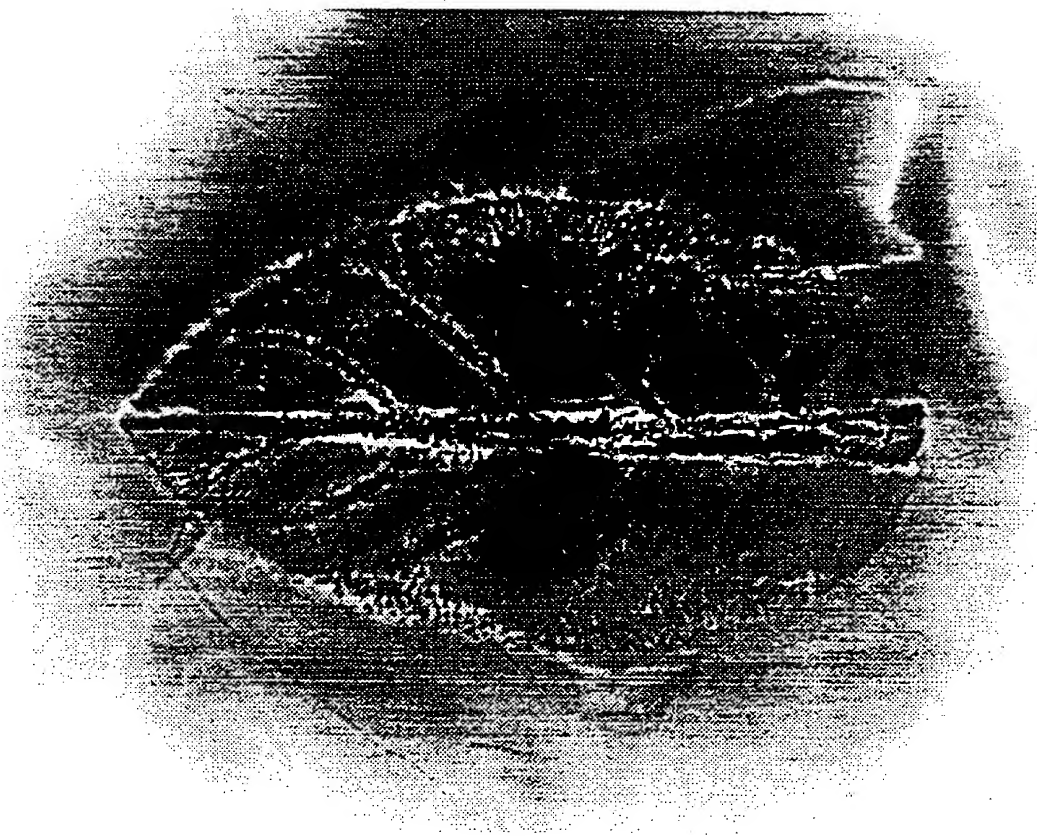


FIGURE 23



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FIGURE 24



SEQUENCE LISTING

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<130> PPD 50364 GENE SWITCH

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<151> 1998-08-13

<160> 24

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24

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attggatttt tgtcacacta ttgtatcgct gggaatacaa ttacttaaca taagcacctg 180

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Fragment

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40

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 15 20 25

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 30 35 40 45

gcg atc att tat cct cat tcc atg gtt aaa tct gat att tca att cta 194
 Ala Ile Ile Tyr Pro His Ser Met Val Lys Ser Asp Ile Ser Ile Leu
 50 55 60

gat aat tac cct aaa aaa tgg agg caa tat tat gat gac gct aat tta 242
 Asp Asn Tyr Pro Lys Lys Trp Arg Gln Tyr Tyr Asp Asp Ala Asn Leu
 65 70 75

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 Ile Lys Tyr Asp Pro Ile Val Asp Tyr Ser Asn Ser Asn His Ser Pro
 80 85 90

att aat tgg aat ata ttt gaa aac aat gct gta aat aaa aaa tct cca 338
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 Asn Val Ile Lys Glu Ala Lys Ser Ser Gly Leu Ile Thr Gly Phe Ser
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ttc cct att cat act gct aat aat ggc ttc gga atg ctt agt ttt gca 434
 Phe Pro Ile His Thr Ala Asn Asn Gly Phe Gly Met Leu Ser Phe Ala
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 35 40 45

Tyr Pro His Ser Met Val Lys Ser Asp Ile Ser Ile Leu Asp Asn Tyr
 50 55 60

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Lys Asp Asn Tyr Ile Asp Ser Leu Phe Leu His Ala Cys Met Asn Ile
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Ala Asn Asn Lys Ser Asn Asn Asp Leu Thr Lys Arg Glu Lys Glu Cys
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 195 200 205

Leu Gly Cys Ser Lys Arg Thr Val Thr Phe His Leu Thr Asn Ala Gln
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Phe Asn Gln Ser Gly Asn Ile Ala Asp Ser Ser Leu Ser Phe Thr Phe
          15             20             25

act aac agt agc aac ggt ccg aac ctc ata aca act caa aca aat tct 147
Thr Asn Ser Ser Asn Gly Pro Asn Leu Ile Thr Thr Gln Thr Asn Ser
          30             35             40

caa gcg ctt tca caa cca att gcc tcc tct aac gtt cat gat aac ttc 195
Gln Ala Leu Ser Gln Pro Ile Ala Ser Ser Asn Val His Asp Asn Phe
          45             50             55             60

atg aat aat gaa atc acg gct agt aaa att gat gat ggt aat aat tca 243
Met Asn Asn Glu Ile Thr Ala Ser Lys Ile Asp Asp Gly Asn Asn Ser
                65             70             75

aaa cca ctg tca cct ggt tgg acg gac caa act gcg tat aac gcg ttt 291
Lys Pro Leu Ser Pro Gly Trp Thr Asp Gln Thr Ala Tyr Asn Ala Phe
                80             85             90

gga atc act aca ggg atg ttt aat acc act aca atg gat gat gta tat 339
Gly Ile Thr Thr Gly Met Phe Asn Thr Thr Thr Met Asp Asp Val Tyr
          95             100             105

aac tat cta ttc gat gat gaa gat acc cca cca aac cca aaa aaa gag 387
Asn Tyr Leu Phe Asp Asp Glu Asp Thr Pro Pro Asn Pro Lys Lys Glu
          110             115             120

ggt ggg tcg acc ccg gga att cag atc cta aca atg ggt atg aaa gac 435
Gly Gly Ser Thr Pro Gly Ile Gln Ile Leu Thr Met Gly Met Lys Asp
          125             130             135             140

ata aat gcc gac gac act tac aga ata att aat aaa att aaa gct tgt 483
Ile Asn Ala Asp Asp Thr Tyr Arg Ile Ile Asn Lys Ile Lys Ala Cys
                145             150             155

aga agc aat aat gat att aat caa tgc tta tct gat atg act aaa atg 531
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 270 275 280

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 Asp Ser Leu Phe Leu His Ala Cys Met Asn Ile Pro Leu Ile Val Pro
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Gln Pro Ile Ala Ser Ser Asn Val His Asp Asn Phe Met Asn Asn Glu
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Ile Thr Ala Ser Lys Ile Asp Asp Gly Asn Asn Ser Lys Pro Leu Ser
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 Gly Met Phe Asn Thr Thr Thr Met Asp Asp Val Tyr Asn Tyr Leu Phe
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 115 120 125
 Pro Gly Ile Gln Ile Leu Thr Met Gly Met Lys Asp Ile Asn Ala Asp
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 Asp Thr Tyr Arg Ile Ile Asn Lys Ile Lys Ala Cys Arg Ser Asn Asn
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 Asp Ile Asn Gln Cys Leu Ser Asp Met Thr Lys Met Val His Cys Glu
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 Tyr Tyr Leu Leu Ala Ile Ile Tyr Pro His Ser Met Val Lys Ser Asp
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 Asp Ala Asn Leu Ile Lys Tyr Asp Pro Ile Val Asp Tyr Ser Asn Ser
 210 215 220
 Asn His Ser Pro Ile Asn Trp Asn Ile Phe Glu Asn Asn Ala Val Asn
 225 230 235 240
 Lys Lys Ser Pro Asn Val Ile Lys Glu Ala Lys Ser Ser Gly Leu Ile
 245 250 255
 Thr Gly Phe Ser Phe Pro Ile His Thr Ala Asn Asn Gly Phe Gly Met
 260 265 270
 Leu Ser Phe Ala His Ser Glu Lys Asp Asn Tyr Ile Asp Ser Leu Phe
 275 280 285
 Leu His Ala Cys Met Asn Ile Pro Leu Ile Val Pro Ser Leu Val Asp
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<211> 1206

<212> DNA

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Cys Leu Ser Asp Met Thr Lys Met Val His Cys Glu Tyr Tyr Leu Leu													
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65 70 75													
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255 260 265													

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act aac agt agc aac ggt ccg aac ctc ata aca act caa aca aat tct 914
Thr Asn Ser Ser Asn Gly Pro Asn Leu Ile Thr Thr Gln Thr Asn Ser
290 295 300

caa gcg ctt tca caa cca att gcc tcc tct aac gtt cat gat aac ttc 962
Gln Ala Leu Ser Gln Pro Ile Ala Ser Ser Asn Val His Asp Asn Phe
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atg aat aat gaa atc acg gct agt aaa att gat gat ggt aat aat tca 1010
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320 325 330

aaa cca ctg tca cct ggt tgg acg gac caa act gcg tat aac gcg ttt 1058
Lys Pro Leu Ser Pro Gly Trp Thr Asp Gln Thr Ala Tyr Asn Ala Phe
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gga atc act aca ggg atg ttt aat acc act aca atg gat gat gta tat 1106
Gly Ile Thr Thr Gly Met Phe Asn Thr Thr Thr Met Asp Asp Val Tyr
350 355 360 365

aac tat cta ttc gat gat gaa gat acc cca cca aac cca aaa aaa gag 1154
Asn Tyr Leu Phe Asp Asp Glu Asp Thr Pro Pro Asn Pro Lys Lys Glu
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<211> 391

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<400> 10

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Tyr Pro His Ser Met Val Lys Ser Asp Ile Ser Ile Leu Asp Asn Tyr
50 55 60

Pro Lys Lys Trp Arg Gln Tyr Tyr Asp Asp Ala Asn Leu Ile Lys Tyr
65 70 75 80

Asp Pro Ile Val Asp Tyr Ser Asn Ser Asn His Ser Pro Ile Asn Trp
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Asn Ile Phe Glu Asn Asn Ala Val Asn Lys Lys Ser Pro Asn Val Ile
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His Thr Ala Asn Asn Gly Phe Gly Met Leu Ser Phe Ala His Ser Glu
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 195 200 205
 Leu Gly Cys Ser Lys Arg Thr Val Thr Phe His Leu Thr Asn Ala Gln
 210 215 220
 Met Lys Leu Asn Thr Thr Asn Arg Cys Gln Ser Ile Ser Lys Ala Ile
 225 230 235 240
 Leu Thr Gly Ala Ile Asp Cys Pro Tyr Phe Lys Ser Ile Asp Lys Leu
 245 250 255
 Met Pro Lys Lys Lys Arg Lys Val Ser Ser Gly Ala Asn Phe Asn Gln
 260 265 270
 Ser Gly Asn Ile Ala Asp Ser Ser Leu Ser Phe Thr Phe Thr Asn Ser
 275 280 285
 Ser Asn Gly Pro Asn Leu Ile Thr Thr Gln Thr Asn Ser Gln Ala Leu
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 305 310 315 320
 Glu Ile Thr Ala Ser Lys Ile Asp Asp Gly Asn Asn Ser Lys Pro Leu
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<210> 13

<211> 26

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:LasBox1
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<210> 14

<211> 26

<212> DNA

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<211> 25

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:TraBox1
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<210> 16

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<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:TraBox2
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<210> 17

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

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<210> 18
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<220>
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35 40 45
His Ser Met Val Lys Ser Asp Ile Ser Ile Leu Asp Asn Tyr Pro Lys
50 55 60
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Ile Val Asp Tyr Ser Asn Ser Asn His Ser Pro Ile Asn Trp Asn Ile
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Phe Glu Asn Asn Ala Val Asn Lys Lys Ser Pro Asn Val Ile Lys Glu
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115 120 125
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130 135 140
Asn Tyr Ile Asp Ser Leu Phe Leu His Ala Cys Met Asn Ile Pro Leu
145 150 155 160
Ile Val Pro Ser Leu Val Asp Asn Tyr Arg Lys Ile Asn Ile Ala Asn
165 170 175
Asn Lys Ser Asn Asn Asp Leu Thr Lys Arg Glu Lys Glu Cys Leu Ala
180 185 190
Trp Ala Cys Glu Gly Lys Ser Ser Trp Asp Ile Ser Lys Ile Leu Gly
195 200 205
Cys Ser Glu Arg Thr Val Thr Phe His Leu Thr Asn Ala Gln Met Lys

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 50 55 60
 Asp Arg Ala Gly Tyr Ala Arg Val Asp Pro Thr Val Ser His Cys Thr
 65 70 75 80
 Gln Ser Val Leu Pro Ile Phe Trp Glu Pro Ser Ile Tyr Gln Thr Arg
 85 90 95
 Lys Gln His Glu Phe Phe Glu Glu Ala Ser Ala Ala Gly Leu Val Tyr
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 Gly Leu Thr Met Pro Leu His Gly Ala Arg Gly Glu Leu Gly Ala Leu
 115 120 125
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 Glu Ser Val Leu Pro Thr Leu Trp Met Leu Lys Asp Tyr Ala Leu Gln
 145 150 155 160
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 165 170 175
 Leu Thr Ser Arg Glu Lys Glu Val Leu Gln Trp Cys Ala Ile Gly Lys
 180 185 190
 Thr Ser Trp Glu Ile Ser Val Ile Cys Asn Cys Ser Glu Ala Asn Val
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 <213> *Agrobacterium tumefaciens*

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 35 40 45
 Ala Val Thr Asn Tyr His Arg Asp Trp Arg Ser Ala Tyr Phe Glu Asn
 50 55 60
 Asn Phe Asp Lys Leu Asp Pro Val Val Lys Arg Ala Lys Ser Arg Lys
 65 70 75 80
 His Val Phe Ala Trp Ser Gly Glu Gln Glu Arg Ser Arg Leu Ser Lys
 85 90 95
 Glu Glu Arg Ala Phe Tyr Ala His Ala Ala Asp Phe Gly Ile Arg Ser
 100 105 110
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 Thr Leu Ala Ser Glu Arg Pro Ala Ile Asp Leu Asp Arg Glu Ile Asp
 130 135 140
 Ala Ala Ala Ala Ala Gly Ala Val Gly Gln Leu His Ala Arg Ile Ser
 145 150 155 160
 Phe Leu Gln Thr Thr Pro Thr Val Glu Asp Ala Ala Trp Leu Asp Pro
 165 170 175
 Lys Glu Ala Thr Tyr Leu Arg Trp Ile Ala Val Gly Met Thr Met Glu
 180 185 190
 Glu Val Ala Asp Val Glu Gly Val Lys Tyr Asn Ser Val Arg Val Lys
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<211> 720

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<213> *Pseudomonas aeruginosa*

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<210> 24
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 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: LuxI Box
 Promoter Region

<400> 24
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21

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/02653

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/52 C12N15/82 C12Q1/68 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12Q C07K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 06268 A (ZENECA LTD ;JEPSON IAN (GB); PAINE JACQUELINE ANN MARY (GB)) 20 February 1997 (1997-02-20) see the whole document; pref. p.12-14; examples	1,2,8,9, 11-18, 22-24, 28-34
X	WO 96 37609 A (ZENECA LTD ;JEPSON IAN (GB); MARTINEZ ALBERTO (GB); GREENLAND ANDR) 28 November 1996 (1996-11-28) cited in the application see the whole document; pref. p.5-7; examples --- -/--	1,2,8,9, 11-18, 22-24, 28-34

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date or another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

15 November 1999

Date of mailing of the international search report

22/11/1999

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Kania, T

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/02653

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROBSON N D ET AL: "Bacterial N-acyl-homoserine-lactone-dependent signalling and its potential biotechnological applications" TRENDS IN BIOTECHNOLOGY, GB, ELSEVIER PUBLICATIONS, CAMBRIDGE, vol. 15, no. 11, page 458-464 XP004092668 ISSN: 0167-7799 see esp. p.462 l. par. - p.464; figure 3 ----	1-34
A	US 5 196 318 A (BALDWIN THOMAS O ET AL) 23 March 1993 (1993-03-23) the whole document ----	1-34
A	US 5 759 798 A (DUNLAP PAUL VERNON) 2 June 1998 (1998-06-02) the whole document -----	32

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 99/02653

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